MOLECULAR CLONING AND CHARACTERIZATION OF ARABIDOPSIS 14-3-3 PROTEINS

By

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TABLE OF CONTENTS

page

ACKNOWLEDGMENTS ii

ABSTRACT v

CHAPTERS

1 LITERATURE REVIEW 1

Functions and Associated Activities 1
Structures of 14-3-3 Proteins. 14
Mechanisms of 14-3-3 Mediated Protein-Protein Interactions. 17
Summary on 14-3-3 Functions and Biochemical Properties. 20
Gene Families, Isoform Specificity and Evolution. 21
Concluding Remarks. 23

2 THE HETEROLOGOUS INTERACTIONS AMONG PLANT 14-3-3 PROTEINS AND IDENTIFICATION OF REGIONS THAT ARE IMPORTANT FOR DIMERIZATION 25

Introduction 25
Materials and Methods. 27
Results. 30
Discussion. 45

3 THE ARABIDOPSIS 14-3-3 MULTIGENE FAMILY 50

Introduction 50
Materials and Methods. 53
Results. 55
Discussion. 86

4 CHARACTERIZATION OF TRANSGENIC ARABIDOPSIS PLANTS OVEREXPRESSING ANTISENSE AND SENSE 14-3-3 GENES 91

Introduction 91
Materials and Methods 93
Results and Discussion 94

5 SUMMARY 104

REFERENCES 108

BIOGRAPHICAL SKETCH 120

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14-3-3 proteins participate in and regulate diverse steps of different cellular signal transduction pathways in many eukaryotic organisms. For examples, in mammals, 14-3-3 proteins were shown to bind to and regulate tyrosine hydroxylase, and to be involved in mitogen activated protein kinase pathway, T-cell activation pathways, and apoptosis pathways; in plants, 14-3-3 proteins were found to associate with transcription factor complex, to bind to and inhibit nitrate reductase and to be involved in perception of phytotoxin fusicoccin. In Arabidopsis, sequences of six cDNA and three genomic clones of 14-3-3 proteins were isolated and identified in previous studies.

To further characterize the 14-3-3 multigene family in Arabidopsis, the sequences of four new cDNA and two new genomic clones of 14-3-3 genes were determined. Together with six previously described 14-3-3 isoforms in Arabidopsis, they constitute a complete family of ten distinct 14-3-3 proteins. Phylogenetic analysis revealed the presence of two ancient, distinct 14-3-3 gene classes in Arabidopsis and other plants. In

addition, It was demonstrated that eight Arabidopsis and one maize 14-3-3 protein were able to dimerize with each other and with themselves. Native gel western analysis of Arabidopsis cell extracts also suggested the presence of 14-3-3 heterodimers *in vivo*. The domains that are sufficient for homodimerization and heterodimerization were also identified in GF14 chi. To further examine the functions of Arabidopsis 14-3-3 proteins, transgenic Arabidopsis plants overexpressing sense and antisense 14-3-3 genes were established. These transgenic plants displayed unaltered morphology compared with those of wild-type plants, indicating that further detailed analysis is required to elucidate the nature and extent of the potential alterations caused by these transgenes.

CHAPTER 1
LITERATURE REVIEW

The 14-3-3 proteins were initially isolated from bovine brain as abundant, acidic, soluble proteins (Moore and Perez, 1967). They were originally thought to be neuronal tissue specific, conserved among mammals, and uniquely important to brain and neuronal function (Boston et al., 1982a; Boston et al., 1982b; Erikson and Moore, 1980; Ichimura et al., 1991). However, they have now been shown to be widely distributed and different levels are expressed in most mammalian tissues. In addition, proteins that show a high degree of similarity have been cloned and sequenced from a wide range of eukaryotic species, including plants, animals, and fungi.

Functions and Associated Activities

Animals

Following the initial discovery of 14-3-3 proteins, there were no possible biochemical functions ascribed to them for next twenty years. In 1987, Ichimura and co-workers (Ichimura et al., 1987) discovered that 14-3-3 proteins were similar in amino acid composition to a protein known to activate tyrosine and tryptophan hydroxylases (TRH and TPH, respectively), which are rate-limiting enzymes involved in catecholamine and

serotonin neurotransmitter biosynthesis, respectively, in a phosphorylation-dependent manner (Erikson and Moore, 1980; Yamauchi et al., 1981). Later experiments demonstrated that the biochemical properties of purified 14-3-3 were indistinguishable from those of purified activators of TRH and TPH. Ichimura *et al.* (Ichimura et al., 1988) then went on to purify seven 14-3-3 homologs and also cloned the first 14-3-3 isoform. Each isoform was assigned a Greek letter (alpha-eta) on the basis of their order of elution during reverse-phase high-performance liquid chromatography and the elution profile is highly conserved in mammalian species (Aitken et al., 1992).

To date, the exact mechanism by which 14-3-3 proteins activate TRH and TPH remains unknown. However, later experiments showed that 14-3-3 proteins apparently exerted their influences through binding to phosphorylated TPH (Furukawa et al., 1993; Ichimura et al., 1995) and all bovine isoforms, as well as one plant 14-3-3 homolog, GF14 omega, could also activate TPH (Isobe et al., 1991; Lu et al., 1994a). These studies illustrate two characteristics in following 14-3-3 research since the initial functional discovery. First, 14-3-3 proteins associate with other proteins, of which most, if not all, are phosphorylated proteins; second, 14-3-3 proteins from diverse species seem to share similar fundamental properties.

14-3-3 proteins have also been implicated as potential regulators of phopholipid/Ca^{2+}-dependent protein kinase C (PKC). However, there have been contradictory results from studies that analyzed the effects of 14-3-3 on PKC activities *in vitro*. Some reported that 14-3-3 proteins mediate the inhibition of PKC activity that can be overcome by high concentration of diacylglycerol (Robinson et al., 1994; Toker et al.,

1990; Toker et al., 1992), others described the phosphatidylserine or diacylglycerol-independent activation by 14-3-3 (Isobe et al., 1992; Lu et al., 1994a). Still others failed to find any direct effects of 14-3-3 on PKC (Chen and Wagner, 1994; Morgan and Burgoyne, 1992a). In spite of these conflicting data, the interaction of 14-3-3 proteins with PKC is well established and the contradictory reports may reflect the use of different analysis conditions, different preparation of 14-3-3 proteins and/or different PKC isozymes being used. Most recently, using 14-3-3 tau isoform and a Ca^{2+}-independent PKC theta which both are abundantly expressed in T cells, Meller and colleagues were able to, for the first time, demonstrate that 14-3-3 tau directly interacts with PKC *in vitro* and *in vivo* and that 14-3-3 inhibits the function of PKC theta by preventing its translocation to the membrane and by merely associating with it (Meller et al., 1996). Thus, in this case, one particular 14-3-3 isoform does appear to inhibit a PKC isoform activity *in vivo*.

Another function associated with 14-3-3 proteins is Ca^{2+}-dependent stimulation of exocytosis in adrenal chromaffin cells (Morgan and Burgoyneb, 1992; Roth et al., 1993). Because the stimulation is mediated by Ca^{2+} and PKC, it was thought that the 14-3-3 proteins may directly activate PKC to start the process that eventually leads to the exocytosis. However this conclusion is complicated by the data suggesting that Exo 1 (a cytosolic member of the 14-3-3 family) cannot directly activate PKC. The exact mechanism of this activity remains unclear.

Since 1994, there have been numerous reports that described the interactions of 14-3-3 proteins with different components of diverse receptor-initiated signal transduction

pathways, for instance, mitogen activated protein kinase (MAPK) pathway, T cell activation pathway, insulin like growth factor I receptor initiated pathway, adhesion receptor initiated pathway, and apoptosis pathway. These reports suggest that 14-3-3 proteins play roles in cell proliferation, differentiation and death signaling processes in mammals. Many of these pathways appear to be able to cross-talk to each other through 14-3-3 proteins and/or other proteins.

To date, the involvement of 14-3-3 proteins in the MAPK cascade has been the most intensely studied area in 14-3-3 biology. Activation of the MAPK pathway is a central event in the response of cells to mitogenic factors and in many cases is dependent on activated Ras. Raf-1 binds to activated Ras and this interaction recruits Raf-1 to the plasma membrane (PM) where it is activated by an unknown mechanism (Leevers et al., 1994; Stokoe et al., 1994).

14-3-3 proteins interact with Raf-1. The evidence supporting this interaction are compelling and have been the subject of many reviews (Aitken, 1995; Ferl, 1996; Morrison, 1994). In summary, 14-3-3 proteins associate with Raf-1 in yeast two-hybrid system (Freed et al., 1994; Irie et al., 1994) and in binding assays *in vitro* (Fu et al., 1994) and in immunoprecipitate of Raf-1 expressed in insect cells and from mammalian cells (Freed et al., 1994; Fu et al., 1994).

In spite of the convincing data demonstrating the interaction between Raf-1 and 14-3-3, the functional aspects of this interaction remain less clear. Initially, 14-3-3 proteins were shown to activate Raf-1 *in vivo* (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994; Shimizu et al., 1994). Later, however, 14-3-3 proteins were also

shown to associate with inactive Raf-1 in cytosol (Freed et al., 1994; Fu et al., 1994), and *in vitro*, 14-3-3 proteins fail to activate Raf-1. Recently, two independent groups discovered that increased concentration of Raf-1, recruited by activated Ras at the PM, will induce oligomerization of Raf-1 which, in turn, leads to the activation of Raf-1 (Farrar et al., 1996; Luo et al., 1996). These data, together with a previous report that suggested that 14-3-3 dimerization might be a precondition of Raf-1 activation (Luo et al., 1995), indicate that 14-3-3 proteins may play an auxiliary role during Raf-1 activation by facilitating Raf-1 oligomerization (two Raf-1 molecules are associated with each 14-3-3 dimer).

14-3-3 proteins may also play a role of maintaining the active state of Raf-1. They were shown to block the inactivation/dephosphorylation of purified Raf-1 (Dent et al., 1995; Muslin et al., 1996) *in vitro* and this function can be similarly carried out by hsp90 (Dent et al., 1995). Therefore, 14-3-3 proteins probably exert their effects on Raf-1 activation by, first, promoting the oligomerization of Raf-1 and then helping stabilize the activity or conformation of activated Raf-1 kinase, a function similar to that of a chaperone.

14-3-3 proteins have also been shown to interact with B-raf, a homolog of Raf-1, in the MAPK pathway, in the yeast two-hybrid system (Papin et al., 1996). The physiological significance of this interaction remains unknown.

Another activity ascribed to 14-3-3 proteins is their ability to activate the ADP ribosylation activity of Exoenzyme S, a virulence factor associated with infection by *Pseudomonas aeruginosa* (Fu et al., 1993). Later experiments showed that a plant 14-3-3

homolog can substitute for 14-3-3 zeta in the assay, indicating this property is likely conserved in all 14-3-3 proteins (Chen et al., 1994; Lu et al., 1994a). The facts that Ras and other small guanosine triphosphate-binding proteins can be ADP-ribosylated and the activated Ras can bring Raf-1 and 14-3-3 into proximity suggest an intriguing hypothesis that Raf-1 bound 14-3-3 may induce the ribosylation of Ras. However, there has been no report showing Ras can be ribosylated *in vivo*.

Bcr and Abl genes encode serine/threnonine and tyrosine kinase activities, respectively. The first exon of *bcr* is fused upstream of the second exon of *abl* and form a chimeric oncogene, encoding a fusion protein BCR-ABL which is characteristic of certain leukemias (Witte, 1993). BCR and BCR-ABL interact with 14-3-3 proteins through the domain encoded by the first exon of Bcr and this interaction appears to contribute to the oncogenecity of BCR-ABL and is required for full transforming ability. Recently, Braselmann and McCormick (Braselmann and McCormick, 1995) reported that BCR and Raf-1 form a complex *in vivo* via 14-3-3 proteins. The first exon of Bcr also interacts with another protein–Grb2 adapter protein which is a component in many signal transduction pathways, including MAPK pathway and T cell antigen receptor TCR•CD3-initiated signal transduction pathway (see below). These data suggest that 14-3-3 dimer (see below Structures) may, like Grb2, play an adapter/modulator role assembling different proteins into a ternary complex.

In support of the adapter hypothesis, Conklin *et al.* (Conklin et al., 1995) showed that 14-3-3 proteins associate with cdc25A and B phosphatases which play key roles in cell cycle progression by activating cyclin-dependent kinases. cdc25 phosphatases also

associate with Raf-1, which stimulates their phosphatase activity (Galaktionov et al., 1995). The association between 14-3-3 and cdc25 phosphatase, however, has no effect on the phosphatase activity of cdc25A. Nonetheless, interactions between Raf-1, cdc25 phosphatases, and 14-3-3 proteins probably connect the cell cycle control pathway with the mitogenic signaling pathway.

14-3-3 proteins were also found to associate with the middle tumor antigen (MT) of murine polyomavirus (Pallas et al., 1994). MT is known to interact with many proteins which are involved in regulation of cell proliferation, including pp60c-Src, phosphatidylinositol 3-kinase (PI3-K), protein phosphatase 2A, Src homologs and collagen protein and growth factor receptor-binding protein 2. PI3-K, which plays an important role in protein tyrosine kinase (PTK) coupled mitogenic and other signaling pathways, was later shown to interact with and be inhibited by 14-3-3 tau *in vivo* (Bonnefoy-Berard et al., 1995). Whether MT, PI3-K and 14-3-3 tau can form a ternary complex *in vivo* remains unknown.

Cbl is an oncogene associated with myeloid and lymphoid leukemains in mice. It encodes a 160 kDa protein that is a major PTK substrate in TCR•CD3-activated T cells (Donovan et al., 1994; Meisner et al., 1995). 14-3-3 tau and zeta interact with serine phosphorylated Cbl *in vivo* and *in vitro* and the interaction is significantly increased after the T cell activation, in contrast to those between 14-3-3 proteins and Raf-1 which is not affected by T cell activation (Liu et al., 1996; Liu et al., 1997). Interestingly, tyrosine phosphorylated Cbl also associates with PI3-K in an activation-dependent manner and Cbl constitutively associates with Grb2. Therefore, the TCR•CD3-initiated signal transduction

pathway may cross-talk with the MAPK pathway through Cbl, Grb2, PI3-K and 14-3-3 proteins.

Recently, 14-3-3 proteins were shown to interact with several receptors directly. One of the receptors is Insulin-like growth factor I receptor (IGFIR). Craparo and co-workers (Craparo et al., 1997) demonstrated, using the yeast two-hybrid system and *in vitro* interaction assays, that 14-3-3 epsilon protein associated with IGFIR and insulin receptor substrate I. However, the physiological significance of these interactions remains unknown.

14-3-3 zeta binds to the cytoplasmic domain of the adhesion receptor, glycoprotein Ib-IX in platelets. (GPIb-IX) (Du et al., 1996). Zeta was initially purified and cloned as a platelet intracellular phospholipase A_2 (Loeb and Gross, 1986; Zupan et al., 1992). This phospholipase function was later discounted for both animal and plant isoforms (Robinson et al., 1994). The 14-3-3 zeta is relatively abundant in platelets and has been shown to both be associated with the PM and present in the cytosol (Loeb and Gross, 1986). However, purified 14-3-3 zeta does not bind to phospholipid vesicles (Roth et al., 1994). Therefore, association with GPIb-IX may account for at least part of the membrane-associated fraction of this protein.

BAD is distant member of BCL2 family of proteins which constitutes the first of the decision points within a common cell-death pathway (Farrow and Brown, 1996). BAD exerts its death-promoting effects by heterodimerizing with BCL-2 or BCL-X_L death antagonists (Yang et al., 1995). Phosphorylated BAD associates with 14-3-3 proteins in the cytosol, whereas only nonphosphorylated BAD heterodimerizes with BCL-X_L at a

membrane site (Zha et al., 1996). Thus, these data suggest that 14-3-3 may play a modulating role in apoptosis. In support of this notion, 14-3-3 proteins were also found to be associated with A20 *in vitro* and *in vivo* (Vincenz and Dixit, 1996). A20 is an inhibitor of tumor necrosis factor-induced apoptosis (Opipari et al., 1992). Raf-1 and A20 co-immunoprecipitate in a 14-3-3-dependent manner (Vincenz and Dixit, 1996). In addition, upon co-transfection of 14-3-3 and A20, the cellular localization and subcellular fractionation of A20 is drastically altered from insoluble cytoplasmic structures to the soluble cytoplasmic compartment, suggesting 14-3-3 proteins may act as solubilizing chaperones. Other evidence suggesting 14-3-3 proteins as solubilizing factor comes from the studies by Liao and Omary (Liao and Omary, 1996). They showed that 14-3-3 proteins associate with simple epithelial keratins K8/K18 in a phosphorylation-dependent manner, and 14-3-3 proteins sequester phosphorylated K8/18 in soluble fraction *in vitro*. These latest reports again strengthen the idea that 14-3-3 proteins may act as adapter/modulator/chaperone in diverse signal transduction pathways.

14-3-3 proteins are also implicated to be involved in regulation of protein trafficking. Mitochondrial import Stimulation Factor (MSF) is a cytoplasmic chaperone from rat liver that seems to help target protein precursor to the mitochondria (Hachiya et al., 1994). MSF was found to be a heterodimer of rat zeta and epsilon (Alam et al., 1994).

14-3-3 proteins can themselves be phosphorylated by various kinases at tyrosine/or serine residues and phosphorylation may influence their activities. In brain, alpha and sigma are the *in vivo* phosphorylated forms of beta and zeta, respectively (Aitken et al., 1995a). And sigma isoform interacts more strongly with PKC than zeta isoform does

(Aitken et al., 1995b). The tau isoform from human is phosphorylated at serine(s) by Bcr and at tyrosine by Bcr-Abl (Reuther et al., 1994). Sheep brain isoforms (Toker et al., 1992) and plant 14-3-3 (Lu et al., 1994b) homolog, GF14 omega, all can be phosphorylated at serine(s).

14-3-3 proteins from mammals and plants have been shown to bind Ca^{2+} (Aitken, 1995; Ferl, 1996; Morrison, 1994). The functional consequences of this interaction remain unknown. Certain mammalian isoforms (gamma and epsilon) were shown to bind to phospholipid and aggregate phospholipid vesicles (Roth et al., 1994). However, crystal structures of 14-3-3 proteins do not indicate any membrane-spanning motif (see below). Thus, it is likely that these 14-3-3 proteins associate with the membrane by interacting with other membrane proteins.

Yeast

Yeast appears to have only two 14-3-3 isoforms; BMH1 and BMH2 in budding yeast (*Saccharomyces cerevisiae*) and *rad*24 and *rad*25 in fission yeast (*Schizosaccharomyces pombe*) (Ford et al., 1994; van Heusden et al., 1995). Both budding yeast and fission yeast appear to have signaling elements homologous to those in the mammalian MAPK pathway (Errede and Levin, 1993). Thus, BMH1 and 2, and *rad*24 and 25 may perform similar functions as their counterparts in mammals in regard to MAPK pathway.

In budding yeast, overexpression of one of the 14-3-3 homolog BMH1 depresses growth (van Heusden et al., 1992). The double mutant of BMH1 and BMH2 is lethal and

Arabidopsis GF14 omega can complement the double mutant (van Heusden et al., 1995). Overexpression of BMH1 enhances the function of mammalian Raf-1 in yeast and BMH1 is required for the Ras-mediated Raf-1 activation (Irie et al., 1994). Multiple copy BMH2 can rescue the deletion mutant of the clathrin heavy-chain gene (CHC1) (Gelperin et al., 1995). CHC1 deletion causes growth, morphological and membrane trafficking defects in budding yeast (Payne and Lemmon, 1994). High-copy BMH1 can also rescue CHC1 deletion, although not as effectively as BMH2 (Gelperin et al., 1995).

In fission yeast, 14-3-3 homologs *rad*24 and *rad*25 act as DNA damage check point control during mitosis (Ford et al., 1994). Although serving similar function, *rad*24 mutant produces more drastic effects that that of *rad*25, indicating *rad*24 as the major player in DNA damage check point control. Similar to BMH1 and BMH2 double mutant, *rad*24 and *rad*25 double mutant is inviable.

It is clear that even in the relatively simple yeast system, different 14-3-3 proteins seem to perform multiple functions and unique isoforms appear to show certain degree of functional specialization.

Plants

The first activity attributed to plant 14-3-3 homologs is that of association with G-box binding complex. G-box is a cis-element involved in stress and ABA activation of genes. G-box factors (GBF) are a family of bZIP transcription factors that bind to G-box element *in vitro* and *in vivo*. In search of protein factors associated with G-box binding

complex, several monoclonal antibodies that can specifically supershift the G-box-protein complex were raised against partially purified G-box-protein in Arabidopsis (Lu et al., 1992). Using those antibodies, Lu and co-workers isolated five isoforms of 14-3-3 proteins and they were named GF14 (G-box factor 14-3-3 homolog) (Lu et al., 1992).

The ability of 14-3-3 proteins to associate with transcription complexes apparently is conserved in many plant species. Anti-GF14 antibodies can supershift diverse G-box related extracts, including those from maize (de Vetten et al., 1992) and tobacco (Chen et al., 1994) and two 14-3-3 isoforms were isolated from maize using similar methods (de Vetten et al., 1992).

Plant 14-3-3 proteins do not interact with G-box element directly, as indicated by the absence of DNA binding motif in their deduced amino acid sequences and the failure of *E. coli* expressed GF14 proteins to bind G-box element (unpublished results). In addition, 14-3-3 proteins do not interact directly with GBF, either *in vitro* or in the yeast two-hybrid system (unpublished results). Thus, 14-3-3 proteins may associate with G-box binding complex via other mechanism(s), perhaps through interacting with unidentified protein(s) containing 14-3-3 binding consensus sequence(s) (see below).

Plant 14-3-3 proteins have been shown to be a part of the receptor (or receptor complex?) for the phytotoxin fusicoccin (FC) (Korthout and de Boer, 1994; Marra et al., 1995; Oecking et al., 1994). FC, secreted by the pathogen *Fusicoccum amygdali*, is the causative agent of pathological wilting in several plant species. FC receptor (FCR), a membrane-bound protein, can activate H^+-ATPase upon binding of fusicoccin. FCR was purified to homogenecity and sequencing of peptide digests of the purified receptor

reveals that it consists of a member (or members) of the 14-3-3 protein family (Korthout and de Boer, 1994; Marra et al., 1995; Oecking et al., 1994).

There have been contradictory data regarding the localization of FCR and the relationship between 14-3-3 proteins and FCR. One group reports that FCR is localized on the outer PM (de Boer et al., 1987), while others showed that FCR resides on the cytosolic side of the PM (Aducci et al., 1980; Feyerabend and Weiler, 1989; Rubinstein, 1982). As to whether 14-3-3 proteins by themselves can bind FC, some groups suggest the 14-3-3 proteins as *bona fide* FCR, based on the good correlation between the amount of (^{3}H) FC-binding and that of purified 14-3-3 proteins (Korthout and de Boer, 1994; Oecking et al., 1994), while others claim that the involvement of other factor(s) are required for FC binding (Aducci et al., 1995). Nonetheless, the facts that sequences of 14-3-3 proteins predict no membrane-spanning domain (confirmed by crystal structures, see below), and non-permeating macromolecular FC-derivatives are able to bind to receptors (Aducci et al., 1980; Feyerabend and Weiler, 1988), and 14-3-3 proteins exist in all plant species examined, while FC binding activity is limited to only several species, suggest that 14-3-3 proteins alone are unlikely to bind to FC. It is plausible that 14-3-3 proteins may associate with the PM via some unidentified proteins in certain plant species and facilitate FC binding activity through these interaction(s).

Plant 14-3-3 proteins have also been shown to associate and inhibit nitrate reductase (Bachmann et al., 1996a; Bachmann et al., 1996b; Moorhead et al., 1996). NAD(P)H:nitrate reductase (NR) catalyzes the first step in nitrate assimilation, the reduction of nitrate to nitrite in plant species. NR is highly regulated by both molecular

and genetic control of the amount of NR and rapid control of its enzymatic activity through reversible protein phosphorylation (Crawford, 1995; Lillo, 1994; Solomonson and Barber, 1990). The regulation of NR by phosphorylation involves two steps. First, NR is phosphorylated at Ser-543 by a Ca^{2+}-dependent kinase. Second, phosphorylated NR binds to an inhibitor protein (IP) and produces an inactive complex (Bachmann et al., 1995; Glaab and Kaiser, 1995; MacKintosh et al., 1995). The IP was later identified as a mixture of 14-3-3 proteins (Bachmann et al., 1996a; Moorhead et al., 1996). To date, NR represents the only protein identified from plant origin that interacts directly with 14-3-3 proteins and inhibition of NR activity is the only definitive biochemical function ascribed to plant 14-3-3 proteins. Recombinant Arabidopsis 14-3-3 proteins can bind to and inhibit the dephosphorylation/activation of native NR from spinach *in vitro* (Bachmann et al., 1996b), suggesting that the ability to bind and inhibit NR by 14-3-3 proteins is probably conserved across plant species.

Structures of 14-3-3 Proteins

The crystal structures of mammalian 14-3-3 tau and zeta have been solved (Liu et al., 1995; Xiao et al., 1995). They share very similar structural features and are compatible with the results from previous biochemical analysis.

14-3-3 proteins exist as dimers. Each monomer in the 14-3-3 dimer consists of nine alpha helices. These monomers then associate to form dimers (Fig. 1-1) which

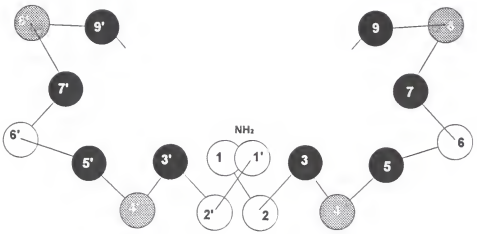


Figure 1-1. Schematic representation of 14-3-3 dimer architecture adapted from Xiao et al. (1995). The highly conserved alpha helices are shaded.

resemble a groove or channel. The dimer interface is formed by packing the helix alpha1 from one monomer against the helices alpha3 and alpha4 from the other (Liu et al., 1995).

There are several distinguishing features of this groove structure. First, non-conserved residues are generally not involved in the packing of alpha helices, thus enabling different 14-3-3 sequences to fit nicely into this model. Second, most of the residues lining inside the groove are conserved across species, suggesting that this surface may recognize certain common motifs shared by different proteins (see below). Third, residues from helices alpha3, alpha5, alpha7 and alpha9 form a amphipathic groove in which, on one side, alpha7 and alpha9 present a hydrophobic surface and, on the other side, alpha3 presents three basic side chains (K49, R56 and R60) and alpha5 presents polar and charged groups. The non-conserved residues on the outer surface of the groove may also bind to specific proteins. Therefore, the groove-shape 14-3-3 dimer may serve as a docking clamp in assembly of diverse protein complexes.

Diverse 14-3-3 proteins from both mammals and plants can form heterodimers via their N-terminal domains, as determined by various biochemical studies (Jones et al., 1995a; Wu et al., 1997a). If different 14-3-3 proteins can bind to different proteins (see below), then heterodimers clearly will add another level of regulation to the protein complex formation.

Mechanisms of 14-3-3 Mediated Protein-Protein Interactions

As more and more proteins are reported to associate with 14-3-3 proteins, interests in the nature of 14-3-3-protein interactions have increased. Several observations suggest that these interactions are phosphorylation-dependent. First, all 14-3-3-protein interactions were described in eukaryotic expression systems, suggesting that proper modification, perhaps phosphorylation, of 14-3-3 proteins or their partners is required for these associations. Second, the interaction between 14-3-3 proteins and tyrosine hydroxylase can only occur after TRH is phosphorylated by threonine/serine kinase calmodulin kinase (Furukawa et al., 1993). Third, phosphatase treatment of Raf-1 and BCR inhibits their interactions with 14-3-3 proteins *in vitro* (Michaud et al., 1995) and 14-3-3 association with Raf-1 can block the ability of phosphatase to inhibit Raf-1 (Dent et al., 1995). Based on the above observations, Muslin and co-workers, using a variety of methods, including the surface plasmon resonance and peptide competition, identified a phosphoserine consensus sequence, RSX_PSXP, to which different 14-3-3 proteins equally bind with high affinity (Muslin et al., 1996). Comparison of the sequences of proteins known to interact with 14-3-3 proteins revealed that many contain sequences similar to the consensus motif (Table 1-1). Later reports confirmed that similar sequences are responsible for interactions with 14-3-3 proteins from both plant and mammalian origins. However, some proteins without this consensus sequence clearly bind to 14-3-3 proteins, likely via other motifs (Table 1-1).

Sequences in different 14-3-3 proteins sufficient for binding to diverse proteins were also identified. Through extensive deletion analysis, Luo and colleagues showed that

Table 1-1. List of known 14-3-3 binding proteins and their putative binding sequences.

Raf-1	R^{256}STSTP
Raf-1	R^{618}SASEP
B-RAF	R^{243}SSSAP
B-RAF	R^{607}SASEP
PKC epsilon	R^{343}SKSAP
PKC gamma	R^{686}SPSSP
Polyoma middle T antigen	R^{270}SHSYP
CDC25B	R^{303}SPSMP
BCR	R^{368}SQSQN
TRH	R^{266}HASSP
BAD	R^{109}SRSAP
BAD	R^{133}HSSYP
A20	R^{562}SKSDP
NR	R^{540}TASTP
p85 subunit of PI3-K	R^{228}SPSIP
GPIb-IX	R^{604}YSGHS
Cbl	R^{617}HSLPFS
Cbl	R^{626}LGSTFS
IGFIR	S^{1272}ASSSSLP
IRS	K^{638}SVSAP
K8/K18	N.A.
PKC theta	N.A.

N.A. not available

C-terminal 65 amino acid (alpha helix 8 and alpha helix 9) of 14-3-3 zeta (total 245 amino acids) are sufficient to mediate binding to Raf-1 and only full-length 14-3-3 sequences are found in association with active Raf-1 (Luo et al., 1995). Using similar methods, Ichimura and coworkers determined that amino acids 171-213 (alpha helix 7 and alpha helix 8) in C-terminal region of 14-3-3 eta bind to phosphorylated TPH that contains a phosphoserine consensus sequence (Ichimura et al., 1995). Of note, this region is conserved among species, from mammals, yeast to plants. Therefore, it is very likely that diverse 14-3-3 proteins can bind to similar motifs via this conserved sequence.

N-terminal sequences are also found to be critical for the protein-binding capability of 14-3-3 proteins. Liu *et al* (Liu et al., 1996) described that the deletions of N-terminal sequence of 12 and 30 amino acid (alpha helix 1 and alpha helix 2), which abolish the dimerization, also disrupt the interaction of 14-3-3 tau with Raf-1, Cbl, and p85 subunit of PI3-K. Using various point mutants of 14-3-3 zeta, Zhang and co-workers determined that the positive charge of K49 is required for the interactions between full-length 14-3-3-zeta and different ligands (Zhang et al., 1997). Hence, it is possible that both N- and C-terminal sequences are directly involved in binding to various proteins. It is also conceivable that some N-terminal residues, such as K49, may provide initial contact points for 14-3-3-protein interactions. Upon the initial contact, a conformation change is induced

Some proteins bind to 14-3-3 proteins through multiple loci that contain the phosphoserine consensus sequence. Raf-1 has two such loci, Ser^{259} and Ser^{621}, which can both bind to 14-3-3 when phosphorylated (Muslin et al., 1996). However, only Ser^{621} is constitutively phosphorylated, therefore 14-3-3 proteins seem to bind to this site only in

unstimulated cells. Ser^{259} appears to be phosphorylated upon Raf-1 activation. Therefore, 14-3-3 proteins probably bind to this site after Raf-1 activation. Association of 14-3-3 proteins with activated Raf-1 apparently blocks the dephosphorylation of phosphotyrosine in active Raf-1 protein, therefore maintaining the active state of Raf-1 (Jelinek et al., 1996). 14-3-3 also divalently bind to BAD and A20, respectively (Vincenz and Dixit, 1996; Zha et al., 1996). The functional consequences of this two-site binding are unclear.

Summary on 14-3-3 Functions and Biochemical Properties

Numerous reports suggest that 14-3-3 proteins are involved in diverse signal transduction pathways in mammalian, yeast, and plant species. A certain degree of consensus has been forming regarding the fundamental properties of these proteins. 14-3-3 apparently exert its influences through interacting with proteins with similar motifs. Many of 14-3-3 interacting proteins are components of disparate signal transduction pathways that are critical for cell growth and proliferation.

Through association with 14-3-3 dimers, some proteins, many of which are protein kinases and/or kinase substrates, may come into proximity where phosphorylation or other modifications may occur. These proteins include Raf-1, Bcr, A20 etc. (Braselmann and McCormick, 1995; Vincenz and Dixit, 1996). Other proteins, such as Raf-1 kinase (Jelinek et al., 1996), nitrate reductase (Bachmann et al., 1996b) and perhaps tyrosine hydroxylase, may stabilize their phosphorylated state upon interacting with 14-3-3 proteins. These interactions can result in either activation or inhibition of the interacting

proteins. Still, the association between 14-3-3 proteins and some proteins apparently affect the subcellular localization of these proteins, such as PKC (Meller et al., 1996), BAD (Zha et al., 1996), and A20 (Vincenz and Dixit, 1996), and therefore affect the functions of those proteins, and sometimes, the fate of the cell (Zha et al., 1996).

The mechanisms of 14-3-3-protein interactions are basically understood. And it appears that these mechanisms are likely to account for most, if not all, 14-3-3-protein interactions, making it one of the most significant fundamental properties probably shared by all 14-3-3 isoforms (see below). This common characteristics and possibly other properties may explain the reason why 14-3-3 proteins from diverse origins can substitute each others in various *in vitro* and *in vivo* assays.

Gene Families, Isoform Specificity and Evolution

14-3-3 proteins are encoded by gene families in all eukaryotic species examined. In mammals, seven isoforms have been identified (Aitken et al., 1992). In plants, multiple isoforms have been cloned from many species and Arabidopsis possesses the largest complete 14-3-3 gene family of ten isoforms (Wu et al., 1997b).

Why do eukaryotic organisms have multiple isoforms which seem to share similar properties? One appealing hypothesis is that different isoforms may specialize in different functions via their unique intrinsic properties and/or distinct expression patterns and/or differential responses to environmental cues.

Several lines of evidence suggest that different isoforms may possess distinct intrinsic properties. First, studies of yeast 14-3-3 homologs (see above) showed that different 14-3-3 isoforms apparently encode for distinct functions in two-isoform yeast system despite their high homology. Second, some mammalian 14-3-3-interacting proteins, such as Raf-1 and A20, appear to preferentially co-immunoprecipitate with certain 14-3-3 isoforms (Vincenz and Dixit, 1996). Third, spinach nitrate reductase seems to associate with recombinant Arabidopsis 14-3-3 proteins in a isoform-specific manner *in vitro* (Bachmann et al., 1996b).

The distinct expression profile of different isoforms in mammals and plants may also attribute to their specific functions. In mammals, certain isoforms, such as tau and sigma, do show cell-specific expression (Leffers et al., 1993; Martin et al., 1993; Martin et al., 1994; Nielsen, 1991; Roth et al., 1994). In neuronal tissues, where 14-3-3 proteins were initially identified, most isoforms show certain degree of overlapping and distinct expression pattern (Watanabe et al., 1994). In Arabidopsis, characterized isoforms demonstrate cell-preferential expression patterns as determined by northern blot analysis (chapter 4). Different plant 14-3-3 isoforms also respond distinctively to differential environmental stimuli. Some Arabidopsis isoforms are induced under low temperature (Jarillo et al., 1994); some maize isoforms appear to be induced by hypoxia (de Vetten and Ferl, 1995); while one rice homolog is regulated by both salt and low temperature (Kidou et al., 1993) and one barley isoform is induced after pathogen attack (Brandt et al., 1992). These observations suggest the involvement of plant 14-3-3 isoforms in diverse signaling pathways.

Sequence analysis of different 14-3-3 isoforms revealed that they are highly conserved except the extreme N- and C-terminal regions (Ferl et al., 1994; Wang and Shakes, 1996), suggesting all 14-3-3 proteins may share similar properties. This in accordance with findings that demonstrate different 14-3-3 proteins bind to proteins containing the phosphoserine consensus sequence.

Various evolution studies of 14-3-3 sequences suggest that mammal and plant 14-3-3 lineages have been on divergent evolutionary tracts since their early separation (Ferl et al., 1994; Wang and Shakes, 1996; Wu et al., 1997b), indicating there could be no shared specialized functions between plant and mammalian 14-3-3 proteins.

In mammals, various isoforms were well established before mammals and possibly vertebrates diverged (Wang and Shakes, 1996). In contrast, most plant isoforms appear to arise after the separation of two major lineages, epsilon-like and non-epsilon isoforms (Wu et al., 1997b).

Concluding Remarks

In spite of the bewildering arrays of activities associated with the 14-3-3 proteins, our understanding of the fundamental properties of this class of proteins has greatly improved over the past few years. These proteins, apparently by binding to phosphoserine containing sequences, interact with a huge number of diverse proteins and may act as either adapter and/or chaperone and/or modulator, depending on the particular target proteins.

The complete understanding of exact functional impacts of different 14-3-3 proteins in particular species, even in certain cells, still begs new discoveries of 14-3-3-interacting proteins and thorough characterization of the interactions and their effects. Identification and characterization of 14-3-3-interacting protein mutants that abolish the ability to interact with 14-3-3 proteins while preserving full functional activities will give us greater understanding about the functions of these 14-3-3 proteins. Conversely, defining the phenotypes of 14-3-3 single and/or multiple mutants will lend insight into which biochemical pathways individual isoform(s) participate. Alternatively, detailed characterization of the phenotype of transgenic organisms carrying one or multiple antisense genes may also serve the same purpose. Finally, with regard to the global impacts of 14-3-3 proteins in particular species, extensive characterization of transgenic organisms with overexpression of dimerization domain of 14-3-3 proteins may provide valuable implications that will likely lead to the greater understanding of entire 14-3-3 protein family.

CHAPTER 2
THE HETEROLOGOUS INTERACTIONS AMONG PLANT 14-3-3 PROTEINS AND IDENTIFICATION OF REGIONS THAT ARE IMPORTANT FOR DIMERIZATION

Introduction

The 14-3-3 proteins are a family of proteins whose members have particular migration patterns on two dimensional "protein maps" made by DEAE-cellulose chromatography and starch gel electrophoresis (Moore and Perez, 1967). The 14-3-3 family is highly conserved at the amino acid sequence level over a wide range of species including mammals, plants and yeast (Aitken et al., 1992; Ferl et al., 1994). However, there are several regions, including the amino- and carboxy termini, that have rapidly diverged to give each isoform a unique sequence (Ferl et al., 1994). The 14-3-3 proteins are involved in a diverse range of cellular functions, many through protein-protein interaction, such as binding and activation of tyrosine and tryptophan hydroxylases (Ichimura et al., 1988; Ichimura et al., 1995), regulation of protein kinase C activity (Aitken et al., 1990; Toker et al., 1990; Isobe et al., 1992), exocytosis from adrenal chromaffin cells (Morgan and Burgoyne, 1992b) and regulation of cell cycle in fission yeast (Ford et al., 1994). Recently, mammalian 14-3-3 beta and zeta have been found to associate with Raf-1 kinase, a key member in the MAP kinase cascade (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al., 1994). The mammalian 14-3-3 tau isoform

has been shown to bind and be phosphorylated by Bcr kinase (Reuther et al., 1994). Mammalian 14-3-3 isoforms have also been shown to associate with polyoma virus middle T antigen, which in turn associates with other proteins involved in the regulation of cell proliferation (Pallas et al., 1994).

These findings raise questions of how such a simple, highly conserved protein is involved in such diverse functions in different species. One explanation is that different 14-3-3 homologs can interact with each other to form diverse heterodimers capable of participating in various cellular functions. In mammals the structure and dimerization characteristics of two 14-3-3 proteins, zeta and tau, have been well studied. The X-ray crystallographic structures of 14-3-3 zeta and tau indicate that both monomers consist of nine antiparallel alpha-helices with the first four helices contributing to the dimeric interface (Liu et al., 1995; Xiao et al., 1995). Cross-linking and immuno-precipitation show that the N-terminal half of the sheep 14-3-3 epsilon isoform is able to mediate homodimerization and heterodimerization with the 14-3-3 zeta isoform (Jones et al., 1995a). However, whether the heterodimerization among 14-3-3 proteins is promiscuous, whether heterodimerization between diverse isoforms requires different domains of 14-3-3 proteins and whether heterodimers really exist within the cell remain to be elucidated.

Lu et al. (Lu et al., 1992) discovered a family of 14-3-3 proteins in Arabidopsis through immuno screening of a cDNA expression library, using a monoclonal antibody that recognizes a component of G-box DNA binding complex. These proteins were designated GF14 proteins (G-box Factor 14-3-3 homologs) (Lu et al., 1992). In this study, I demonstrate that all known Arabidopsis isoforms exist as dimers and that they can

form heterodimers among themselves, with three newly isolated Arabidopsis 14-3-3 homologs, and with a maize 14-3-3 protein (de Vetten et al., 1992). Native gel western analysis of Arabidopsis suspension cell extract also indicates the possibility of 14-3-3 heterodimers *in vivo*. Finally, the domains of a 14-3-3 protein that are able to mediate dimerization among different 14-3-3 proteins were identified.

Materials and Methods

Reagents

Restriction endonucleases and reagents for polymerase chain reaction (PCR) were obtained from Boehringer-Mannheim (Germany). pET15b vector and reagents for protein expression and purification were from Novagen (Madison, WI) and Boehringer-Mannheim. Reagents for protein gel electrophoresis were from Boehringer-Mannheim and Sigma (St. Louis, MO). Western blot reagents were obtained from Schleicher&Schuell (Keene, NH) and Amersham (Little Chalfont, England). Superdex 75 column and reagents for DNA nested deletion were from Pharmacia (Piscataway, NJ). Yeast alcohol dehydrogenase protein (ADH) and reagents for *in vitro* heterodimerization assays were from Sigma. MATCHMAKER™ vectors and yeast cell HF7c were purchased from Clontech (Palo Alto, CA). Reagents for yeast culture media were from Difco (Detroit, MI) and Sigma.

Expression and Purification of GF14 proteins

The Arabidopsis GF14 proteins were expressed and purified as previously described (Lu et al., 1994a). The coding sequences of GF14 proteins were amplified by PCR using synthetic oligodeoxynucleotides and subcloned into the NdeI-BamHI site of pET15b. All 14-3-3 clones are in frame as determined by sequencing. The pET15b vector provided the expressed GF14 proteins with a histidine tract at their amino termini, allowing for purification by Ni^{2+}-charged immobilized metal affinity chromatography (IMAC) according to the manufacturer's protocol (Novagen). The histidine tract was removed by human thrombin cleavage, dialysis, and IMAC. The purified proteins were dialyzed in NEB buffer (20 mM Hepes, 40 mM KCl, 0.1 M EDTA, 10% glycerol, 5 mM BME, pH7.5) and the protein concentration was determined by the Bradfrod microassay (Bio-Rad, Richmond, CA) with BSA as standard.

Gel Filtration Chromatography

Ten μg of each purified GF14 protein were loaded on a Superdex 75 column in degassed buffer containing 50 mM Tris pH7.5, 100 mM NaCl and 1 mM NaN_3. The sample was flowed through the column at a speed of 0.5 ml/min. Elution fractions were collected and subjected to SDS polyacrylamide gel electrophoresis (PAGE).

In vitro Heterodimerization Assays by Native Gel Electrophoresis

For *in vitro* heterodimerization assays, two equal amounts (2 μg each, total volume of 4 μl) of GF14 purified proteins or an addition of yeast ADH (10 μg, volume of

10 μl in NEB buffer) were mixed at 4 ^{0}C. The mixture was denatured by dialyzing in 6 M guanidine hydrochloride NEB (6 M guanidine hydrochloride, 20 mM Hepes, 40 mM KCl, 0.1 M EDTA, 10% glycerol, 5 mM BME, pH7.5) for 3 hours and then renatured by dialyzing in 3 M guanidine hydrochloride NEB, 1 M guanidine hydrochloride NEB for 1 hour each and 0.1 M guanidine hydrochloride NEB and NEB for 2 hours each. The protein mixtures were analyzed on a 10% non-denaturing polyacrylamide gel (0.375 M Tris, pH 8.8). After electrophoresis, the gel was stained with Coomassie Blue.

Western Blot Analysis

Western blot analysis was performed as previously described (Lu et al., 1992). Arabidopsis suspension cell extract was prepared as previously described (23,24). The total number of bands on native Western analysis was counted by vision.

Yeast Two-Hybrid Assays

Coding regions of GF14 isoforms were amplified by PCR and subcloned into a Bam HI site of each of the two-hybrid shuttle/expression vectors pGBT9 and pGAD424. All coding regions are in frame as determined by sequencing. Various combinations of two-hybrid constructs were co-transformed into the yeast cell HF7C according to the protocol provided by the manufacturer (Clontech). Equal amounts of transformant were spread on two dropout media, one deficient in leucine, trytophan and histidine and another one deficient in leucine and trytophan. After growing for 4 days at 30 ^{0}C, colonies were counted on both types of medium. Histidine reporter gene activity was calculated as

percentage of yeast colony number on leucine, tryptophan, histidine deficient minimum medium divided by that on leucine, trytophan deficient medium.

Nested Deletion

pGBT chi was first digested with Pst I and then with Sal I. A double-stranded nested deletion series was created by DNase I digestion of double digested pGBT chi according to the protocol by Pharmacia. Deletion sites in the clone were identified by sequencing.

DNA Sequencing

DNA sequencing was performed at the DNA Sequencing Core laboratory at the University of Florida (Gainesville, FL), using fluorescent dye termination sequencing chemistry on an Applied Biosystems 373A DNA sequencer. DNA sequences were analyzed using GeneWorks 2.2 (IntelliGenetics, Mountain View, CA).

Results

Arabidopsis 14-3-3 Proteins Exist As Dimers

To further elucidate the biochemical properties of Arabidopsis 14-3-3 proteins, five GF14 isoforms were expressed as His-tag fusion proteins in *E. coli*. The expression and purification of GF14 chi are shown in Figure 2-1. After thrombin digestion to remove the His-tag, the purified protein is about 99% pure as judged by Coomassie blue staining

Figure 2-1 SDS-PAGE of Expressed GF14 chi Protein.

Lane 1, 3 μl of uninduced cell lysate; Lane 2, 3 μl of IPTG induced cell lysate; Lane 3, 4 μg of Ni^{2+} column eluted GF14 chi protein; Lane 4, 2 μg purified GF14 chi protein after thrombin digestion to remove the histidine tag. Samples were run on 10% SDS polyacrylamide gel and stained with Coomassie blue. Size markers are indicated on the right.

Figure 2-2 Gel Filtration Chromatography of Recombinant GF14 chi.

Ten μg of recombinant GF14 chi protein was eluted from the Superdex 75 column with a native molecular mass near 69 kDa.
(A) The elution peaks for beta-galactosidase (GAL, 116 kDa), bovine serum albumin (BSA 69 kDa) and ricin A chain (RTA, 30 kDa) are shown above the A280 trace for GF14 chi elution peak. The fraction numbers are indicated below the GF14 chi elution peak.
(B) SDS-PAGE analyses of peak fractions show a monomer with molecular mass of about 32 kDa. 5 μl of elution fraction was run on each lane in a 10% SDS-PAGE. The lane numbers correspond to the fraction numbers in panel A. The gel was stained with Coomassie blue after electrophoresis.

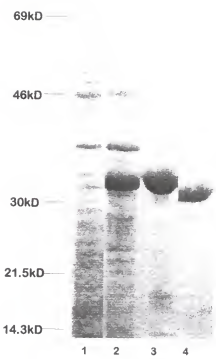


Figure 2-1

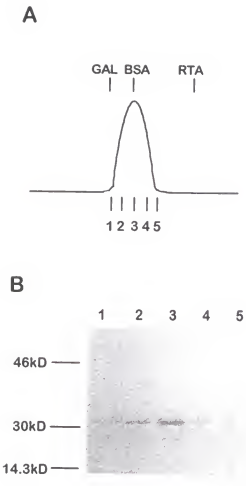


Figure 2-2

after SDS-PAGE. The recombinant GF14 chi protein shows a molecular weight of 32 kDa, 2 kDa larger than the 30 kDa predicted by the amino acid sequence. This is a general characteristic of 14-3-3 proteins including mammalian 14-3-3 homologs (Jones et al., 1995b).

To investigate whether recombinant plant 14-3-3 isoforms exist as dimers, I performed gel filtration chromatography to determine the size of the 14-3-3 proteins under native conditions (Figure 2-2A, only GF14 chi is shown). The elution peak of purified GF14 chi is about the same as that of BSA, a 69 kDa protein, indicating that under native conditions recombinant GF14 chi protein exists as dimer.

Four other GF14 proteins, GF14 phi, GF14 omega, GF14 psi and GF14 upsilon were also expressed and purified in the same manner as GF14 chi. Gel filtration confirmed that they also exist as dimers (data not shown).

In vitro Heterodimerization Among Plant 14-3-3 Proteins Demonstrated By Native PAGE

Figure 2-3 demonstrates that each of the purified recombinant Arabidopsis GF14 proteins shows a distinct mobility on native PAGE. GF14 psi shows a single band of rapid mobility (Figure 2-3A, lane 1). GF14 omega, chi and phi migrate as single bands of increasingly reduced mobility (Figure 2-3A, lanes 2-4). GF14 upsilon shows multiple bands, some of which have similar mobility as that of GF14 chi (Figure 2-3A, Lane 5). The GF14 upsilon protein preparation also has multiple bands on SDS-PAGE, all of which immuno-react with the anti-GF14 antibody (data not shown). Thus the multiple bands are probably due to different translation start sites on the GF14 upsilon mRNA in *E. coli*. A

Figure 2-3 Coomassie Blue-Stained Native PAGE of *In Vitro* Specific Heterodimerization Between GF14 Proteins.

(A) Purified recombinant GF14 proteins (2 μg each lanes 1-5) and mixtures of two purified proteins (2 μg each lanes 6-14) which underwent the denaturing and renaturing process as described in materials and methods. After native PAGE the gel was stained for total protein with Commasie Blue. Proteins and protein mixtures are indicated on the top of each lane.
(B) Heterodimer formation between GF14 psi and GF14 chi proteins cannot be disrupted by yeast ADH (10 μg). Heterodimers were found in the presence of excess yeast ADH (lane 4) to show that the presence of other proteins have no effect on the heterodimerization between 14-3-3 proteins. GF14 proteins and protein mixtures are indicated on top of the gel. All samples were electrophoresised on 10% native PAGE and stained with Coomassie blue after running. Heterodimer formed between GF14 chi and GF14 chi is indicated with a H. The smear on top of some lanes is probably caused by incomplete refolding of GF14 proteins.

A
GF14 psi
GF14 chi
GF14 phi
GF14 omega
GF14 upsilon
GF14 chi+GF14 phi
GF14 chi+GF14 omega
GF14 chi+GF14 upsilon
GF14 phi+GF14 omega
GF14 omega+GF14 upsilon
GF14 phi+GF14 upsilon
GF14 phi+GF14 psi
GF14 psi+GF14 omega
GF14 psi+GF14 upsilon
1 2 3 4 5 6 7 8 9 10 11 12 13 14
H
B
GF14 chi
GF14 psi
GF14 chi+GF14 psi
GF14 chi+GF14 psi+ADH
1 2 3 4

similar phenomenon was also observed for the *E. coli* expression of mammalian 14-3-3 epsilon isoform (Jones et al., 1995b).

To determine their ability to form heterodimers, equal amount of purified GF14 proteins were mixed together, denatured by dialyzing in 6 M guanidine hydrochloride and renatured by gradually dialyzing away the denaturant. The mixture was resolved on native PAGE (Figure 2-3A, 3B). A band with an intermediate mobility appears for all the isoform combinations tested except that between GF14 chi and GF14 upsilon which is difficult to observe because of their similar mobilities (Figure 2-3A, Figure 2-3B). The intermediate bands resulted from mixture involving GF14 upsilon are visible although not as significant as others (Figure 2-3A). This is probably due to the multiple start sites of GF14 upsilon protein product. The formation of bands of intermediate mobilities clearly indicate that 14-3-3 proteins exist as dimers in the native PAGE system and that heterodimerization readily occurs *in vitro*.

To test the specificity of the heterodimerization, I used yeast ADH which is a homotetramer as a competitor in the *in vitro* heterodimerization assay between GF14 psi and GF14 chi. In the presence of excess ADH, GF14 psi and GF14 chi specifically formed three bands while the ADH created a large smear in the lane, demonstrating heterodimerization quantitatively and qualitatively similar to pure GF14 proteins. Thus, the heterodimerization between these two homologs could not be competed by the yeast ADH (Figure 2-3B, Lane 4), indicating this heterodimerization between GF14s is specific.

In vivo Heterodimerization in Yeast Two-hybrid System

The *in vivo* heterodimerization among Arabidopsis 14-3-3 proteins was further demonstrated using a yeast two-hybrid system. Four Arabidopsis 14-3-3 isoforms and a maize 14-3-3 homolog were subcloned into the two-hybrid vectors, pGBT9 and pGAD424 (Figure 2-4A). Different combinations of the constructs were co-transformed into yeast and possible interactions were tested. The 14-3-3 proteins slightly activate the reporter gene when they were cloned as Gal 4 DNA binding domain (pGBT9) fusion protein, perhaps due to the acidic nature of 14-3-3 proteins. The reporter gene was significantly activated when two 14-3-3 proteins were co-transformed into the yeast cell, indicating interaction between the two proteins. The results in Table 2-1 clearly show that all tested 14-3-3 proteins, namely GF14 chi, GF14 psi, GF14 phi and GF14 upsilon can interact with themselves to form homodimers, and with each other to form heterodimers. Interaction between Arabidopsis GF14 proteins and a maize 14-3-3 homolog were also detected in the two-hybrid system (Table 2-2, only GF14 chi shown).

The Potential *In vivo* Heterodimerization in Arabidopsis Extract

To test the possibility that plant 14-3-3 proteins exist as homodimers and/or heterodimers *in vivo*, I performed western blot analysis using protein extract from the Arabidopsis suspension cells, from which the cDNA library of the original five GF14 proteins were isolated (Lu et al., 1992) (Figure 2-5). In Figure 2-5A, proteins were blotted from a SDS-PAGE gel and probed with anti-GF14 antibody. There are five distinct bands recognized by the antibody in Arabidopsis suspension cell protein extract (Figure 2-5A,

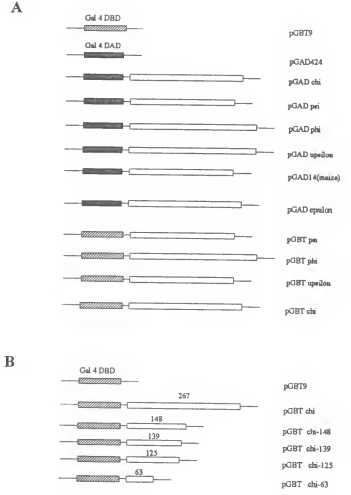


Figure 2-4 Schematic Illustration of Plasmid Constructs Used in the Two-Hybrid System.

(A) Different plant 14-3-3 isoforms were cloned into pGBT9 and pGAD424 two-hybrid vectors (Clontech) as described in materials and methods, except that of GF14 epsilon which is cloned into pGAD10 vector and has extra 16 amino acid on its N-terminal upstream of the first methionine. **(B)** The GF14 chi deletion series were made as described in materials and methods. The numbers above constructs indicate the length of GF14 chi after the deletion. Only the portions that contain the Gal 4 protein binding or activation domain and 14-3-3 constructs are shown.

Table 2-1. Interaction Between 14-3-3 proteins

Construct 2	Construct 1					
		pGAD424	pGAD chi	pGAD psi	pGAD phi	pGAD upsilon
	pGBT9	0 [a]	0	0	0	0
	pGBT chi	9.04 ± 0.98	23.79±2.78	27.32±2.67	27.18±1.65	NA
	pGBT psi	14.23 ±3.10	NA	35.39± 3.24	27.94±1.85	NA
	pGBT phi	10.03 ± 1.10	NA	NA	16.25± 1.07	NA
	pGBT upsilon	8.65 ± 1.24	23.48±2.43	20.18±1.76	16.23± 1.07	20.36±1.76

Construct 1 and construct 2 were cotransformed into yeast cell HF7c. Reporter activity was measured according to Materials and Method.

[a] Relative activity of Histidine reporter are expressed as the mean±SE of three samples

NA Not analyzed.

Table 2-2. Interaction Between GF14 chi Deletion Series and 14-3-3 Proteins

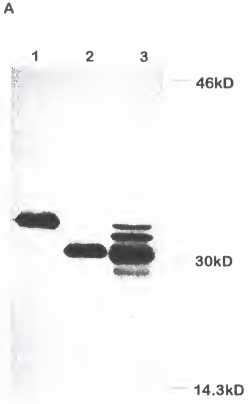
Construct 2	Construct 1: pGAD 424	pGAD chi	pGAD psi	pGAD epsilon	pGAD14(maize)
pGBT9	0 [a]	0	0	0	0
PGBT chi-63	0	0	0	0	0
pGBT chi-125	0	1.60±0.25	8.35± 1.53	0	7.33±1.42
pGBT chi-139	0	20.61±4.39	21.92±3.24	0	12.91± 2.37
pGBT chi-148	0	1.91 ±0.10	7.10 ± 0.47	2.80 ±0.92	9.39 ± 2.14
pGBT chi	9.04±0.98	23.79 ±2.78	27.32±2.67	65.77±4.23	22.12±1.87

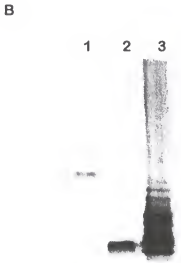
Construct 1 and construct 2 were cotransformed into yeast cell HF7c. Reporter activity was measured according to Materials and Method.

[a] Same as that of Table 2-1.

Figure 2-5 Western Blot Analysis of Protein Extracts of Arabidopsis Hypocotyl Suspension Cell Indicates the Possible Presence of 14-3-3 Heterodimers *In Vivo*.

(A) Western blot from a SDS-PAGE of purified GF14 proteins and Arabidopsis suspension cell extract. Lane 1. 0.05 μg of purified GF14 phi protein. Lane 2. 0.05 μg of purified GF14 psi protein. Lane 3. 20 μg of Arabidopsis suspension cell extract.
(B) Western Blot of a native PAGE . Lane 1. 0.01 μg of purified GF14 phi protein. Lane 2. 0.03 μg of purified GF14 psi protein. Lane 3. 20 μg of Arabidopsis suspension cell extract. The blots were probed by anti-GF14 monoclonal antibody.


A
1
2
3
46kD
30kD
14.3kD


B
1
2
3

Lane 3). Purified GF14 phi and GF14 psi proteins, which have the highest and lowest predicted molecular weights among five isoforms, respectively, were included as markers. The mobility difference between recombinant and native GF14 proteins may be caused by post-translational modification of the latter. In Figure 2-5B, the same samples were run on a native PAGE gel, blotted on a nitrocellulose membrane and probed with anti-GF14 antibody. The suspension cell extract gives rise to at least 15 bands that can be clearly counted, indicating the likely presence of heterodimers *in vivo*. The smear up to the well that is present in the native western while absent in SDS western may reflects the additional association of GF14 proteins with other proteins.

Recovery of New GF14 Isoforms Using the Dimerization Interface

During a yeast two-hybrid screening of Arabidopsis cDNA library using GF14 chi as bait, three new 14-3-3 homologs were isolated which can interact with GF14 chi. They were designated as GF14 epsilon, GF14 kappa and GF14 lambda (Wu et al., 1997a). All five original GF14 proteins can interact with these three newly isolated isoforms in the yeast two-hybrid system (Table 2-2, only GF14 chi and GF14 epsilon shown). A strong interaction was detected between GF14 chi and GF14 epsilon. This strong interaction may be caused by the N-terminal extension of GF14 epsilon that arises from the translation of 5' untranslated region in the yeast two-hybrid GF14 epsilon cDNA clone. This is in contrast to the five original GF14 proteins which were expressed from the first methionine. A strong interaction was also observed for other 14-3-3 isoforms that have artificial N-terminal extensions in the yeast two-hybrid vector. The reason why 14-3-3

proteins with N-terminal extensions have stronger interaction with GF14 proteins remains unknown. One possibility is that N-terminal extensions interfere with the homodimerization while having little or no effect on their heterodimerization with other 14-3-3 proteins, therefore increasing the sensitivity of reporter gene detection.

Dimerization Domain Mapping

To identify domain(s) of GF14 chi that are sufficient for dimerization, C-terminal deletions of GF14 chi were made. They were cloned into the yeast two-hybrid vector pGBT9 (Figure 2-4B), and possible interactions between different deletions and diverse GF14 proteins were tested using yeast two-hybrid system. Table 2-2 shows that all deletions have significantly reduced background compared with full length GF14 chi (268 amino acids), probably because of the removal of the acidic carboxy terminus. pGBT chi-63 which contains the first 63 amino acids of GF14 chi could not interact with any 14-3-3 protein tested. pGBT chi-125 and pGBT chi-139 which retain the first 125 and 139 amino acids, respectively, are able to interact with GF14 chi, GF14 psi and a maize GF14 protein, but not with GF14 epsilon. pGBT chi-148 which has 23 more amino acids on its amino terminus than pGBT chi-125 was able to interact with all 14-3-3 isoforms tested, indicating GF14 chi needs more N-terminal region to form a heterodimer with GF14 epsilon than to form a homodimer and some heterodimers.

Discussion

I show here that five recombinant Arabidopsis GF14 proteins exist as dimers, that they can heterodimerize with each other *in vitro*, and that they are able to interact with three newly isolated Arabidopsis 14-3-3 proteins and a maize 14-3-3 protein to form *in vivo* heterodimers in a yeast two-hybrid system. The *in vitro* heterodimers formed after denaturation and renaturation of 14-3-3 protein mixtures exhibit similar intensity as those of homodimers in the same mixture judged by the Coomassie blue staining (Figure 2-3A, 3B). Therefore 14-3-3 proteins must use a similar mechanism, such as random association of monomers during renaturation, for both heterodimerization and homodimerization. This conclusion is further supported by the results from the two-hybrid system (Table 2-1). X-ray crystal structure analysis of two different mammalian 14-3-3 proteins, zeta and tau, shows that the two 14-3-3 protein monomers dimerize to form a groove with residues making up the dimer interface largely conserved (Liu et al., 1995; Xiao et al., 1995). Provided the high amino acid conservation among 14-3-3 proteins, a similar structure is anticipated for all members of this family. Thus, based on the theory derived from previous studies of mammalian 14-3-3 proteins and on our direct observations with plant 14-3-3 proteins, I predict that all 14-3-3 proteins can promiscuously heterodimerize with each other.

Heterodimers could potentially increase the repertoire of 14-3-3 protein functions. Reports of various 14-3-3 proteins physically associated with different proteins suggest a potential functional specificity of different 14-3-3 proteins as molecular adapters that

mediate the formation of specific protein complexes in diverse cellular processes (Aitken et al., 1990; Braselmann and McCormick, 1995; Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Ichimura et al., 1988; Irie et al., 1994; Isobe et al., 1992; Liu et al., 1995; Pallas et al., 1994; Reuther et al., 1994; Xiao et al., 1995). The ability to form heterodimers would potentially enable 14-3-3 proteins to act as specific molecular adapters in different cellular processes.

In support of this notion, the apparent functional forms of some 14-3-3 proteins are heterodimeric. Results from one group studying the plant fusicoccin receptor seem to indicate that one functional component of fusicoccin receptor, which is a 14-3-3 homolog, exists as heterodimer (Korthout and de Boer, 1994). Mitochondrial import stimulation factor was shown to be a 14-3-3 heterodimer (Hachiya et al., 1994). Because all 14-3-3 proteins can potentially interact with one and another, and like Arabidopsis, the expression patterns of many mammalian 14-3-3 proteins overlap (Aitken et al., 1992), there are ample opportunities for heterodimer formation that would increase the complexity of available 14-3-3 proteins.

I have shown that the N-terminal half of one plant 14-3-3 protein is critical for dimerization. The first 125 amino acids of GF14 chi, which correspond to the first four alpha-helices in the animal 14-3-3 crystal structure, are able to facilitate homodimerization and heterodimerization with some other isoforms (Table 2-2). This result is consistent with the crystal structures of mammalian 14-3-3 proteins which suggest the first four alpha-helices are involved in homodimerization and the mapping of interaction domains of mammalian 14-3-3 isoforms (Jones et al., 1995a; Liu et al., 1995; Xiao et al., 1995). The

GF14 chi deletion that retains the first 65 amino acids which correspond to the first two alpha-helices (pGBT5-63) could not interact with a full length GF14 chi or any other 14-3-3 homologs (Table 2-2), indicating the third and fourth alpha-helices are absolutely required for stable dimerization.

The evolutionarily divergent GF14 epsilon requires a larger domain of GF14 chi for a stable interaction. The extra domain needed for heterodimerization with GF14 epsilon corresponds to the fifth alpha-helix in the crystal structure (Figure 2-6). When aligned with other 14-3-3 homologs, GF14 epsilon distinguishes itself by forming a separate branch in the phylogenic tree, away from both animal and plant mainline lineages, suggesting it may be involved in different cellular functions (Wu et al., 1997b). Its divergence was further demonstrated by the fact that *E. coli* expressed GF14 epsilon protein can not be recognized by anti-GF14 monoclonal antibody, which can recognize all other Arabidopsis 14-3-3 isoforms (data not shown). As shown in Figure 2-6, GF14 epsilon has amino acid substitutions at positions that are otherwise conserved on the dimerization interface of other 14-3-3 proteins. This may slightly affect the interaction between monomers of GF14 chi and GF14 epsilon, such that stable heterodimerization requires more amino acids of GF14 chi. No apparent preference of heterodimerization could be detected among plant 14-3-3 homologs that are more closely related (Ferl et al., 1994). The fact that one 14-3-3 isoform needs a larger domain to interact with a isoform from another phylogenic subgroup suggests the possibility of preference in heterodimerization, which may in turn result in preferred protein complex formation in the cellular process.

Arabidopsis and other species have a large number of 14-3-3 proteins that share a lot of similar properties but may be involved in different cellular functions. Further elucidation of these functions begs the isolation of proteins that interact with 14-3-3s and characterization of expression patterns of specific 14-3-3 genes.

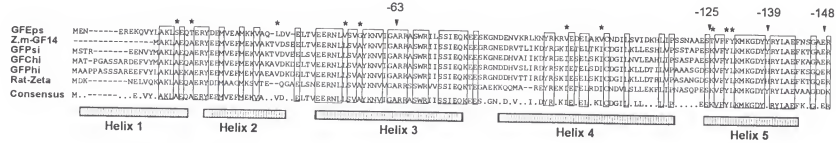


Figure 2-6 Alignment of Amino Termini of 14-3-3 Proteins

Six 14-3-3 proteins from different species are aligned through the dimerization domain of the N-terminal amino acids. The 14-3-3 proteins in the alignment are GF14 epsilon (Wu et al., 1997); GF14 psi, GF14 chi and GF14 phi (Lu et al., 1994); maize GF14 protein (de Vetten et al., 1992) and rat 14-3-3 zeta (Alam et al., 1994). Amino acid residues that are identical among six sequences are boxed, and gaps introduced to optimize the alignment are indicated by dashes. Helix 1 through 5 of the 14-3-3 zeta crystal structure are indicated by bars below the alignment (Liu et al., 1995). The amino acid substitutions that occur only in GF14 epsilon at positions that are otherwise strictly conserved are indicated with asterisks. The arrows and the numbers above the alignment indicate the positions and length of C-terminal deletions of GF14 chi that appear in Figure 2-4 and Table 2-2.

CHAPTER 3
THE ARABIDOPSIS 14-3-3 MULTIGENE FAMILY

Introduction

The 14-3-3 proteins exist as protein families that contain highly conserved, but individually distinct isoforms in many species (Ferl et al., 1994; Ferl, 1996). They were originally isolated as soluble, cytosolic, and acidic proteins from bovine brain on the basis of their electrophoretic mobilities (Moore and Perez, 1967). Further studies showed that 14-3-3 proteins, in addition to their abundance in mammalian brain, are also present in all eukaryotic organisms examined to date (Aitken, 1995; Ferl, 1996).

An assortment of activities and functions have been attributed to 14-3-3 proteins, most of which are involved in cellular signaling pathways. In mammals, there are seven distinct 14-3-3 isoforms. They were initially described as phosphorylation-dependent activators of tyrosine and tryptophan hydroxylase during the biosynthesis of serotonin and dopamine (Yamauchi et al., 1981; Ichimura et al., 1987). Later, 14-3-3 proteins were found to regulate protein kinase C activity (PKC) (Toker et al., 1992; Tanji et al., 1994), activate calcium-dependent exocytosis in permeabilized adrenal chromafin cells (Morgan and Burgoyne, 1992b), and associate with and activate Raf-1 kinase, a key member in the MAP kinase cascade (Fantl et al., 1994; Freed et al., 1994; Fu et al., 1994; Irie et al.,

1994). More recently, mammalian 14-3-3 proteins were shown to bind and be phosphorylated by Bcr kinase (Reuther et al., 1994), associate with polyoma virus middle T antigen, which in turn associates with other proteins involved in the regulation of cell proliferation (Pallas et al., 1994), and stimulate mitochondrial import in rats (Alam et al., 1994). In yeast, the 14-3-3 proteins function as part of the cell-cycle-control DNA-damage check-point in *S. pombe* (Ford et al., 1994) and complex with cdc25 phosphatase (Conklin et al., 1995). In plants, 14-3-3 proteins were found to associate with G-box DNA/protein complex in Arabidopsis and maize (de Vetten et al., 1992; Lu et al., 1992), function as a receptor for the phytotoxin fusiccoccin in certain plants (Korthout and de Boer, 1994; Oecking et al., 1994), and bind and regulate nitrate reductase (Bachmann et al., 1996a, 1996 b; Moorhead et al., 1996). Arabidopsis 14-3-3 proteins were also shown to interchangeably carry out certain cellular functions as ascribed to mammalian and yeast 14-3-3s (Lu et al., 1994a; van Heusden et al., 1995).

At present, the reason why such homologous isoforms are responsible for so many diverse functions remains unknown. One leading model is that different isoforms, while they may have many conserved properties, specialize in individual function(s) on the basis of their isoform diversity, distinct cell-specific and/or developmentally regulated expression, and differential responses to environmental stimuli. In support of this model, some Arabidopsis 14-3-3 proteins demonstrate differential ability to regulate nitrate reductase (Bachmann et al., 1996a)

In Arabidopsis, six 14-3-3 isoforms were identified in previous studies. Five isoforms were isolated during a screening of an expression library using a monoclonal

antibody that recognized component(s) in a G-box DNA/protein complex (Lu et al., 1992; Lu et al., 1994c). They were identified as 14-3-3 proteins based on their amino acid sequence homology with known 14-3-3 proteins and designated as GF14 psi, GF14 chi, GF14 phi, GF14 omega, and GF14 upsilon (**G**-box **F**actor **14**-3-3 homologs). Another isoform, RCI2 (**R**are **C**old-**I**nducible cDNAs), was identified in a screening of cold-acclimated etiolated seedlings of Arabidopsis with a subtracted probe (Jarillo et al., 1994). The genomic clones of three GF14 genes were also identified. They are GRF1-GF14 chi, GRF2-GF14 omega, and GRF3-GF14 psi (**G**eneral **R**egulatory **F**actor, with numbers designating the order in which the genomic clones were identified) (Rooney and Ferl, 1995; Daugherty et al., 1996)

To better understand the functions of 14-3-3 proteins, it is important to fully characterize certain benchmark organisms with respect to all 14-3-3 present. I isolated and characterized four novel 14-3-3 cDNAs in Arabidopsis, namely, GF14 epsilon, GF14 kappa, GF14 mu, and GF14 nu. Together with the six known 14-3-3 isoforms, they represent the largest complete 14-3-3 protein family in all organisms characterized. The isolation and characterization of two new Arabidopsis GF14 genes is also reported here. Sequence analyses of all ten Arabidopsis GF14 proteins, as well as other 14-3-3 proteins, revealed the presence of two divergent classes of Arabidopsis 14-3-3 genes which may encode specialized functions.

Materials and Methods

Isolation of New 14-3-3 Isoforms

Yeast Two-Hybrid Screening

The coding region of GF14 chi was amplified by PCR and subcloned into a Bam HI site of the two-hybrid shuttle/expression vector pGBT9 as previously described (Wu et al., 1997a). The coding region was in frame as determined by sequencing. The construct was co-transformed into HF7C yeast cells with pGAD10 constructs containing a yeast two-hybrid cDNA library of *Arabidopsis thaliana* (Columbia ecotype) to identify potential proteins that could interact with GF14 chi protein, according to the protocol provided by the manufacturer (Clontech, Palo Alto, CA). The transformants were plated on selection media lacking histidine, leucine, and tryptophan. A total of 2×10^6 colonies was screened. Positive colonies that could produce histidine were isolated and DNA were subsequently purified and sequenced.

Searching of Database of Expressed Sequence Tags (dbEST)

Full length cDNA sequences of Arabidopsis GF14 proteins were used as probes during a BLAST search of dbEST of GenBank using the search engine HyperBLAST version 1.10 (NCBI). BLAST search parameters included Expected Number of Matches of 25 and default Scoring Matrix. Arabidopsis EST entries with homology to the probes were retrieved and their sequences were compared with the probes to determine their identity. EST entries that were not identical to any of the already characterized

Arabidopsis GF14 proteins were analyzed to determine unique sequences. Those representing unique 14-3-3 sequences were ordered from the DNA Stock Center of Arabidopsis Biological Resource Center at the Ohio State University and DNA were subsequently purified and sequenced.

Isolation of Genomic Clones of 14-3-3

Isolation of genomic clones of Arabidopsis 14-3-3 genes was carried out as previously described (Daugherty et al., 1996). An *Arabidopsis thaliana* (Columbia ecotype) genomic library (Clontech) was plated on *Escherichia coli* K803 and screened by hybridization at high stringency with ^{32}P-labled GF14 cDNAs. The hybridization was performed at 65^{0}C for 18 hr in 0.5 M sodium phosphate, pH 7.2, 1 mM EDTA, 7% SDS, and 1% BSA. The filters were washed for 1 hr with three changes of 40 mM sodium phosphate, pH 7.2, 24 mM NaCl, 0.1% SDS, and 1 mM EDTA at 65 ^{0}C and exposed to Kodak XAR-5 film with an intensifying screen. Hybridizing recombinant phages were rescreened at lower plaque density to plaque purity. Phage particles were isolated, and DNA were subsequently purified.

Subcloning and Sequencing

Relevant DNA fragments were cloned into pGEM-7Zf(+) vectors and subjected to double-stranded nested deletions (Pharmacia, Piscataway, NJ). The nucleotide sequences of both strands of the plasmid DNA were determined by automated dideoxy chain termination on an ABI 373 (Applied Biosystems, Foster City, CA).

Computational Analyses of 14-3-3 Sequences

Nucleotide and amino acid sequences were managed and alignments were performed using GeneWorks (IntelliGenetics, Mountain View, CA). Amino acid identity of the 14-3-3 proteins and coding nucleotide identity of the 14-3-3 coding sequences were also analyzed with GeneWorks. The neighbor-joining and protein parsimony tree-building programs were provided within version 3.5 of the PHYLIP program package (Felsenstein, 1989).

Low-Stringency Southern Blotting

Genomic DNA was isolated from Arabidopsis suspension cells as described (Ferl and Nick, 1987) and digested with indicated restriction enzymes, subjected to 0.8% agarose gel electrophoresis, and transferred to Hybond-N^+ nucleic acid transfer membrane (Amersham, Arlington Heights, IL). The hybridization and washing were performed in a similar manner as described (see above) except they were carried out at 45^0C.

Results

Isolation and Characterization of Four Novel GF14 Isoforms

During a yeast two-hybrid screening of an Arabidopsis cDNA library using GF14 chi as bait, seven positive clones were identified. Sequencing of those clones revealed three unique 14-3-3 cDNA sequences. Two of the sequences represented novel 14-3-3 proteins. One of the two novel sequences was designated as GF14 kappa. Another one

was designated as GF14 epsilon, based on the results from phylogenetic analysis (see below). One of three sequences was identical to RCI2 (Jarillo et al., 1994). To keep the consistency of nomenclature of Arabidopsis 14-3-3s in our study, RCI2 was designated as GF14 lambda.

To further identify and characterize all members of the Arabidopsis GF14 protein family, a BLAST search of Arabidopsis dbEST was carried out using eight known Arabidopsis GF14 cDNA sequences as probes (Table 3-1), on the basis that all characterized 14-3-3 isoforms were highly homologous. One hundred and three EST entries with homology to the probes were retrieved out of a total of 21,056 Arabidopsis EST entries from GenBank (Oct. 1995). Of the 103 EST entries, only 17 were not identical to any of the probes at the DNA sequence level. Through sequence comparison, the 17 EST entries could be divided into two groups with unique sequences (data not shown). Eight EST clones from these two groups were ordered and sequenced. Sequencing results showed that the two groups represented two new 14-3-3 isoforms. They were designated as GF14 mu and GF14 nu.

As shown in Figure 3-1, each of the four new GF14 cDNAs contains one large open reading frame (ORF) comprising 248 to 265 amino acid residues. The four novel Arabidopsis GF14 proteins contain 5' untranslated leaders of 48 to 68 bp and 3' untranslated regions of 169 to 281 bp, followed by short poly (A^+) tails. There is at least one stop codon in the ORF just upstream of the first ATG on the 5' untranslated leaders of GF14 mu and GF14 nu. This may explain why GF14 mu and GF14 nu could not be identified in an expression library.

Table 3-1. Results of dbEST blast search using Arabidopsis 14-3-3 sequences as probes

14-3-3 Homologs	Match ESTs Accession Numbers
GF14 psi (ψ)	R30193 T45017 T88609 H36612 Z33928 H37251 Z25944 T45464 T42452 H36560 R65375 T44005 T21021 T41947
GF14 chi (χ)	T88244 T42492 Z32630 Z47716 T41737 T21461 Z33784 Z18505 Z29149
GF14 phi (ϕ)	R29957 T43618 T75753 Z26404 Z30732 T42621 Z30731
GF14 omega (ω)	T12954 F13837 F13827 T13740 T04075 T03971 T88062 H36108 R29865
GF14 upsilon (υ)	H37085 T88418
GF14 epsilon (ε)	T20402 Z46557 T88323 T41936 T88028 T44175 T43075 H37302 Z46556
GF14 kappa (κ)	T20470 Z29937 Z34207 T04551 T76574 H37447 T21810 T42214
GF14 lambda (λ)	R64954 R30482 T76099 R36693 F14040 R30460 H36037 T13704 T41754 T41719 R30260 H36731 T42343 R29822 T13629 H36009 F13951 R30565 R64976 T76728 T04438 T75872 T45842 T13899 T41697 T88399 H36742 T88482
GF14 mu* (μ)	T41753 Z25954 T88030 T46314 T21293 R30201 R30326 T76174 T22974 H36714 T76858 H37297 R65479 R65320 Z26527 T22570
GF14 nu* (ν)	T42328

* New 14-3-3 homologs identified through dbEST BLAST search.

Figure 3-1. Nucleotide and deduced amino acid sequences of GF14 epsilon (A), GF14 kappa (B), GF14 mu (C), and GF14 nu (D).

Figure 3-1A

GF14Epsilon

```
GCGGCCGCGTCGACGAAGGAAGAAGAAGAAGAAGAAGAAAAAACTATGGAGAATGAG    60
                                                  M  E  N  E

AGGGAAAAGCAGGTTTACTTGGCTAAGCTCTCCGAGCAAACCGAAAGATACGATGAAATG  120
R  E  K  Q  V  Y  L  A  K  L  S  E  Q  T  E  R  Y  D  E  M

GTGGAGGCGATGAAGAAAGTTGCTCAGCTTGATGTGGAGCTAACTGTGGAAGAGAGGAAT  180
V  E  A  M  K  K  V  A  Q  L  D  V  E  L  T  V  E  E  R  N

CTTGTATCTGTAGGGTACAAGAATGTGATTGGTGCAAGGAGAGCATCATGGAGAATACTA  240
L  V  S  V  G  Y  K  N  V  I  G  A  R  R  A  S  W  R  I  L

TCTTCCATTGAGCAGAAGGAAGAGTCCAAGGGAAATGATGAAAATGTCAAGAGGCTTAAG  300
S  S  I  E  Q  K  E  E  S  K  G  N  D  E  N  V  K  R  L  K

AATTATCGTAAGAGAGTTGAAGATGAGCTTGCTAAAGTTTGTAATGACATCTTGTCTGTC  360
N  Y  R  K  R  V  E  D  E  L  A  K  V  C  N  D  I  L  S  V

ATTGATAAGCATCTCATTCCATCGTCTAACGCTGTGGAGTCAACTGTCTTTTTCTACAAA  420
I  D  K  H  L  I  P  S  S  N  A  V  E  S  T  V  F  F  Y  K

ATGAAAGGAGATTACTATCGCTATCTTGCGGAGTTCAGTTCTGGTGCTGAACGCAAGGAA  480
M  K  G  D  Y  Y  R  Y  L  A  E  F  S  S  G  A  E  R  K  E

GCTGCAGATCAGTCTCTTGAAGCATATAAGGCTGCTGTTGCTGCTGCAGAGAATGGTTTG  540
A  A  D  Q  S  L  E  A  Y  K  A  A  V  A  A  A  E  N  G  L

GCACCCACACATCCAGTTAGACTTGGCTTGGCGTTGAACTTTTCAGTTTTCTACTATGAG  600
A  P  T  H  P  V  R  L  G  L  A  L  N  F  S  V  F  Y  Y  E

ATCTTGAACTCTCCCGAAAGCGCATGCCAATTGGCTAAGCAAGCATTCGATGATGCAATT  660
I  L  N  S  P  E  S  A  C  Q  L  A  K  Q  A  F  D  D  A  I

GCTGAACTTGACAGCCTCAACGAGGAATCATACAAAGACAGCACTCTTATTATGCAGCTA  720
A  E  L  D  S  L  N  E  E  S  Y  K  D  S  T  L  I  M  Q  L

CTTAGAGACAATCTCACCTTGTGGACTTCAGACCTTAATGAGGAAGGAGATGAGAGAACC  780
L  R  D  N  L  T  L  W  T  S  D  L  N  E  E  G  D  E  R  T

AAAGGTGCTGATGAGCCTCAAGATGAGAACTAAATCCTCTGTGAGAAGAGAAACGACTCT  840
K  G  A  D  E  P  Q  D  E  N  *

TGCTGCATCCTGAATCTTGAAGTGAAGACAGTAAGTGTCGTTGTTTGTTACTCGAATGTG  900

TAATTTTTAATCTATGTCTTTCTTGATGGTGTTTTCCAGATTCTTGAACTTTTCACAACA  960

CAACACTGCGTTGCGTATCTTCAACCCTCTTATGATGTGGTTGAATTCTGTTTTACGCTT 1020

AGTTTGCTTCTTTTGTTGTTGAATTGAGCCAGCAGGCATGATTTGGGTTTTTGTTTATCA 1080

GAATATTAGGCGTAAAAAAA                                          1100
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Figure 3-1B

GF14Kappa

```
GCGGGCGCGTCGACCCGATTTCAAAATTTTCCGGTGAAATCGAAAAAAAAAGCGAGATCT      60

TCTTCTCTAATGGCGACGACCTTAAGCAGAGATCAATATGTCTACATGGCGAAGCTCGCC     120
        M  A  T  T  L  S  R  D  Q  Y  V  Y  M  A  K  L  A

GAGCAAGCCGAGCGTTACGAAGAGATGGTTCAATTCATGGAACAGCTCGTAAGTGGAGCT     180
E  Q  A  E  R  Y  E  E  M  V  Q  F  M  E  Q  L  V  S  G  A

ACACCGGCCGGTGAGCTGACCGTAGAAGAGAGGAATCTTCTCTCGGGCGCGTATAAGAAC     240
T  P  A  G  E  L  T  V  E  E  R  N  L  L  S  G  A  Y  K  N

GTGATTGGATCTCTTCGCGCGGCATGGAGAATCGTGTCTTCGCTTGAGCAAAAGGAAGAG     300
V  I  G  S  L  R  A  A  W  R  I  V  S  S  L  E  Q  K  E  E

AGCAGGAAGAACGAAGAACACGTGTCGCTTGTTAAGGATTACAGATCTAAAGTTGAGACT     360
S  R  K  N  E  E  H  V  S  L  V  K  D  Y  R  S  K  V  E  T

GAGCTTTCTTCGATCTTTTCTGGGATTCTCAGGTTACTTGATTCGGATCTAATTCCTTCA     420
E  L  S  S  I  F  S  G  I  L  R  L  L  D  S  H  L  I  P  S

GCTACTGCCAGGGAGTCTAAGGTTTTTTACCTGAAGATGAAAGGAGATTATCATCGTTAT     480
A  T  A  R  E  S  K  V  F  Y  L  K  M  K  G  D  Y  H  R  Y

TTGGCTGAGTTTAAATCTGGTGATGAGAGGAAAACTGCTGCTGAAGATGCTATGATCGCT     540
L  A  E  F  K  S  G  D  E  R  K  T  A  A  E  D  A  M  I  A

TACAAAGCTGCTCAGGACGTTGCAGTTGCTGATCTAGCACCTACACATCCGATCAGGCTT     600
Y  K  A  A  Q  D  V  A  V  A  D  L  A  P  T  H  P  I  R  L

GGTTTGGCTCTTAACTTCTCAGTGTTTTACTACGAGATTCTCAACTCTTCAGAGAAAGCT     660
G  L  A  L  N  F  S  V  F  Y  Y  E  I  L  N  S  S  E  K  A

TGTAGCATGGCGAAACAGGCTTTTGAAGAAGCCATTGCTGAGCTGGACACATTGGGAGAG     720
C  S  M  A  K  Q  A  F  E  E  A  I  A  E  L  D  T  L  G  E

GAGTCATACAAGGACAGTACTCTCATCATGCAGTTGCTAAGGGACAATCTAACCCTTTGG     780
E  S  Y  K  D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W

ACCTCCGATATGCAGGAGCAGATGGATGAGGCCTGAAGGTCTAATGGAAGAAAAGACGGT     840
T  S  D  M  Q  E  Q  M  D  E  A  *

TATGTAATGTACCTGCAACCGTAACCGAAAATCTGAGTTCAACCTCCTTTGCTGTAAAAAC     900

TTGTCGAAAAGAAAAGTTTGTTTTTTTATGACAGATTATGTGCACAGCTTTGGTGTTATC     960

TGCTGCTCTGTATCAACTCTGTTTTTGTTTGGTAATTTATCCTCATCTTTGCTTAAAAGA    1020

AAAAAAAAAAAAAAAAA                                                1037
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Figure 3-1C GF14Mu

```
AGTAATTTAGGTCGTCAAAAGCTTTGGAATTTGATACTTTTGATTTTTCGAGAATCTTGA      60
                                                              *

AAATCAGTCATGGGTTCTGGAAAAGAGCGTGACACTTTCGTCTACCTCGCTAAGCTCTCT     120
        M  G  S  G  K  E  R  D  T  F  V  Y  L  A  K  L  S

GAGCAAGCTGAGCGTTATGAAGAAATGGTGGAATCAATGAAAAGTGTTGCGAAATTGAAT     180
E  Q  A  E  R  Y  E  E  M  V  E  S  M  K  S  V  A  K  L  N

GTTGATCTGACGGTGGAAGAGAGGAACTTACTCTCTGTGGGTTACAAGAACGTGATTGGT     240
V  D  L  T  V  E  E  R  N  L  L  S  V  G  Y  K  N  V  I  G

TCAAGGAGAGCTTCGTGGAGGATCTTCTCGTCGATTGAACAAAAGGAAGCAGTGAAAGGG     300
S  R  R  A  S  W  R  I  F  S  S  I  E  Q  K  E  A  V  K  G

AATGATGTTAATGTAAAGAGGATCAAAGAGTATATGGAGAAGGTTGAGTTAGAGCTTTCT     360
N  D  V  N  V  K  R  I  K  E  Y  M  E  K  V  E  L  E  L  S

AACATATGCATTGATATTATGTCTGTCTTAGATGAGCATCTCATTCCTTCGGCTTCCGAG     420
N  I  C  I  D  I  M  S  V  L  D  E  H  L  I  P  S  A  S  E

GGTGAATCTACTGTCTTCTTCAACAAGATGAAAGGTGACTATTACCGCTATCTTGCTGAG     480
G  E  S  T  V  F  F  N  K  M  K  G  D  Y  Y  R  Y  L  A  E

TTCAAATCAGGGAACGAGAGGAAAGAGGCTGCTGATCAGTCTTTGAAAGCCTATGAGATT     540
F  K  S  G  N  E  R  K  E  A  A  D  Q  S  L  K  A  Y  E  I

GCTACTACTGCTGCTGAGGCTAAGCTCCCTCCAACACACCCTATCAGATTGGGTTTGGCT     600
A  T  T  A  A  E  A  K  L  P  P  T  H  P  I  R  L  G  L  A

TTGAATTTCTCTGTCTTCTACTACGAGATCATGAACGCACCTGAAAGGGCATGTCACCTT     660
L  N  F  S  V  F  Y  Y  E  I  M  N  A  P  E  R  A  C  H  L

GCTAAGCAGGCGTTCGATGAAGCTATCTCAGAGCTTGACACTCTGAGCGAGGAATCCTAC     720
A  K  Q  A  F  D  E  A  I  S  E  L  D  T  L  S  E  E  S  Y

AAAGATAGCACCTTAATAATGCAACTCCTTAGGGACAATCTGACCTTGTGGACTTCTGAC     780
K  D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W  T  S  D

ATCTCAGAAGAAGGAGGAGACGATGCTCATAAGACGAATGGTTCTGCCAAACCTGGTGCT     840
I  S  E  E  G  G  D  D  A  H  K  T  N  G  S  A  K  P  G  A

GGTGGAGACGATGCAGAGTGATATGATATGTGTGCACCTGGACAATATGTTTCAAGAACT     900
G  G  D  D  A  E  *

GAATGTGCGGTGAATAATAGTGAAAAGTAGAGTTTCTCTGTTCCCTATATCATGATTGTC     960

TATGTTACTTGTACTCTGGTTTAGCCCTAAATGTCTCTCTGGTTTGAATGTATTGCATGC    1020

CTGTCTCAGGACACTCTTATTTGTAATTCACTACTGTCGTCCTACTATCTATCCTTATGG    1080

ATCCAATCTTGAAACTAAAAAAAAAAAAAAAAA                               1113
```

Figure 3-1D GF14Nu

```
CCACGCGTCCGCGACAAATCCCTAATTCCAAAATCCTCTCGGATCTAAAAGAGTGTGTGA                60
                                                  *       *

TAACATAAGATGTCGTCTTCTCGGGAAGAGAATGTGTACTTAGCCAAGTTAGCTGAGCAA               120
*       M  S  S  S  R  E  E  N  V  Y  L  A  K  L  A  E  Q

GCTGAACGTTATGAGGAAATGGTTGAGTTCATGGAGAAAGTTGCAAAGACTGTTGACACC               180
A  E  R  Y  E  E  M  V  E  F  M  E  K  V  A  K  T  V  D  T

GATGAGCTTACTGTCGAAGAGAGAAACCTCTTGTCTGTTGCTTACAAGAACGTCATTGGT               240
D  E  L  T  V  E  E  R  N  L  L  S  V  A  Y  K  N  V  I  G

GCTAGGAGAGCTTCCTGGAGGATCATATCTTCCATTGAACAGAAGGAAGAAAGCAGAGGA               300
A  R  R  A  S  W  R  I  I  S  S  I  E  Q  K  E  E  S  R  G

AACGATGATCATGTTTCCATTATCAAGGACTACAGAGGAAAGATCGAAACTGAACTCAGC               360
N  D  D  H  V  S  I  I  K  D  Y  R  G  K  I  E  T  E  L  S

AAAATCTGTGATGGAATACTCAATCTTCTGGATTCTCACCTTGTTCCCACTGCATCTTTG               420
K  I  C  D  G  I  L  N  L  L  D  S  H  L  V  P  T  A  S  L

GCCGAGTCCAAAGTCTTTTACCTCAAAATGAAAGGAGATTACCACAGGTACCTTGCTGAG               480
A  E  S  K  V  F  Y  L  K  M  K  G  D  Y  H  R  Y  L  A  E

TTTAAGACTGGAGCTGAGAGGAAAGAAGCTGCTGAGAGCACTCTGGTTGCTTACAAGTCA               540
F  K  T  G  A  E  R  K  E  A  A  E  S  T  L  V  A  Y  K  S

GCTCAGGATATTGCACTTGCTGATTTAGCTCCTACTCATCCGATTAGACTGGGACTTGCT               600
A  Q  D  I  A  L  A  D  L  A  P  T  H  P  I  R  L  G  L  A

CTTAACTTCTCTGTCTTCTACTACGAGATTCTCAACTCACCTGATCGTGCCTGCAGTCTC               660
L  N  F  S  V  F  Y  Y  E  I  L  N  S  P  D  R  A  C  S  L

GCAAAACAGGCTTTTGATGAGGCCATTTCTGAGCTGGATACATTAGGAGAAGAATCATAC               720
A  K  Q  A  F  D  E  A  I  S  E  L  D  T  L  G  E  E  S  Y

AAAGACAGTACGTTGATAATGCAACTTCTCCGTGACAATCTGACCCTTTGGAACTCTGAC               780
K  D  S  T  L  I  M  Q  L  L  R  D  N  L  T  L  W  N  S  D

ATCAATGATGAGGCGGGCGGTGATGAGATCAAGGAGGCGTCAAAACATGAGCCGGAAGAG               840
I  N  D  E  A  G  G  D  E  I  K  E  A  S  K  H  E  P  E  E

GGGAAACCAGCTGAGACAGGGCAGTGACCAGAGAGAGAGGGAGAGACATTTCTAAGTATG               900
G  K  P  A  E  T  G  Q  *

TATGGTATGGATTTTCCAAAAACATATGATATGATTAGGGGATTTGTAGAAGCAGAGAGA               960

AAGATCTTTATTTGATTTTCTCCAAAAATCTCTGTTCCCTTTTTTTTTATTGGGTTATT              1020

AAAGCTGTTTGAGTCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                         1069
```

The Arabidopsis GF14s encode proteins with calculated molecular weight of 27973 to 30179. The estimated isoelectric of polypeptides range from 4.4 to 4.7 which are similar to those reported for other 14-3-3 proteins (Nielsen, 1991; Brandt et al., 1992; Zupan et al., 1992). The alignment of deduced amino acid sequences of Arabidopsis GF14 proteins is shown in Figure 3-2. The amino acid sequences are highly conserved except in the C-terminal regions. The addition of amino acid sequences of new Arabidopsis GF14 proteins in the alignment does not change the pattern of conserved blocks of amino acids described previously (Ferl et al., 1994). These results are also supported by the observations reported in Table 3-2. As shown in Table 3-2, the percentage of amino acid and coding nucleotide identity of Arabidopsis GF14 proteins range from 60% to 92% and 60% to 86%, respectively.

GF14 Gene Structure and Organization

To characterize the organization and structure of the GF14 genes, an Arabidopsis genomic library in λEMBL3 was screened at high stringency with full length cDNAs of GF14 phi and upsilon. Hybridizing phages were isolated and purified. DNA were subsequently isolated and sequenced. The genomic clones were designated as GRF4-GF14 phi, and GRF5-GF14 upsilon as proposed (Rooney and Ferl, 1995; Ferl, 1996). Their sequences are shown in Figure 3-3.

The genomic structure of the two new Arabidopsis GF14 genes, together with the three that have been previously identified (Rooney and Ferl, 1995), is shown in Figure 3-4. The genomic sequences of GRF1-GF14 chi, GFR2-GF14 omega, and GRF4-GF14 phi reveal three introns in conserved positions (Fig. 3-4); whereas the genomic sequences of

Figure 3-2. Alignment of the entire derived amino acid sequences of the Arabidopsis GF14 proteins. Dashes indicate gaps introduced to facilitate alignment. The numbers on the right indicate the serial numbers of the last residues at the end of GF14 amino acid sequences. The three downward arrows indicate the positions of introns identified in GRF1-GF14 chi, GRF2-GF14 omega, GRF3-GF14 psi, GRF4-GF14 phi, and GRF5-GF14 upsilon gene sequences. The two upward arrows indicate the positions that the amino acid sequences were truncated to facilitate subsequent phylogenetic analysis.

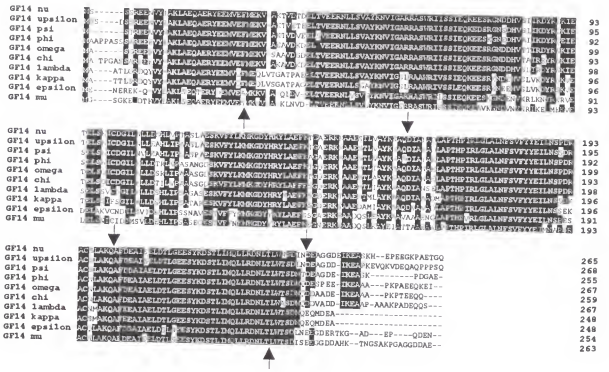

GF14 nu
GF14 upsilon
GF14 psi
GF14 phi
GF14 omega
GF14 chi
GF14 lambda
GF14 kappa
GF14 epsilon
GF14 mu

Table 3-2. Percentage of amino acid and coding nucleotides identity of Arabidopsis GF14s

Comparison was done by the GeneWorks program. Amino acid identity is shown in the the lower part and nucleotide identity in the upper part of the matrix table.

	GF14 nu	GF14 upsilon	GF14 psi	GF14 phi	GF14 omega	GF14 chi	GF14 lambda	GF14 kappa	GF14 epsilon	GF14 mu
						Nuceotide identity				
GF14 nu		83	77	71	71	69	66	65	65	64
GF14 upsilon	89		77	71	69	70	67	67	63	65
GF14 psi	84	84		72	71	71	67	66	65	65
GF14 phi	80	78	79		78	84	68	67	68	64
GF14 omega	82	79	81	89		79	67	67	63	64
GF14 chi	79	77	77	87	89		67	69	60	63
GF14 lambda	69	68	69	71	72	71		86	61	62
GF14 kappa	69	68	69	68	70	69	92		61	62
GF14 epsilon	66	65	69	65	64	63	63	62		68
GF14 mu	67	66	67	64	66	64	60	60	70	
					Amino acid identity					

Figure 3-3. Nucleotide sequence of GRF4-GF14 phi (A) and GRF5-GF14 upsilon (B) gene including introns and 5' and 3' flanking regions.

The nucleotide at position +1 in GRF5-GF14 upsilon corresponds to the first nucleotide of the cDNA. The coding sequence of the genes is bolded. The deduced amino acid sequence is presented below the DNA sequence.

Figure 3-3A GRF4-GF14Phi

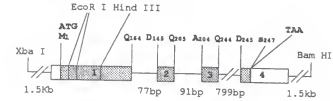
Figure 3-3B GRF5-GF14 upsilon

```
CTAGCTTGCTTACGACATGTCCATACCACAGAAAAAGTTCTTCCTGTTGCGCATTGAGCATACTTGAGACGTACTGATCACATTCCATTAATGCCTTTG      100

CCTTCATCTTGCTACGCAGCGCTAATAACAAAGTATATAGATAATCAATCAAACAACCATTTAACAAAAAAAAACCATCACCATTTCAACTTCAACACAA      200

TCTATAAGCTCAATTCATAGAAGCAATTTGACACACTCAATGAGAGCTTGCTAGATCAGATGCAAAATATGGAGTTACTACCAAGGGTTTATGAGAACCC      300

TAATCCTCAATCTAGCAAAAGAATCTGCGTCTATGATAAGAAACCAGGCTTAAATAGTATCGATATGGTTGATCTCATTTTCGAGGCAATAAAAAACCA      400

TTTGGGGGTTTAGGGTTTAAAACATAGCGAGATGAAGAAGTCACGACATTGTCAAATTACCTTCTTCGACCTTCTTCTTCACGTGATTTTCACGAGCTT      500

GCTCCACATAGCTACCCATTGACGACGGACCTTCGAGCTTTCCGGCCTTCTCGGATCAATTCGATCAAAAGGGCAAAGGGGACTAGAAATGGGTTCAGG      600

GGAAAGGGTAAATCCGTCATTTACCCCAAGGAAACTTAACTTATATTGCCATTTGCCCCTTAATATTTTGTATTATATGAATGACCTTATTAACCTTA      700

TGATTCGTCACATTTCACCCACTTCAATTTAAAATTATGGACGAGAATTAAGTCCTTTGACGGTCAACAGATTAGCACGTGTCGTATTTTAATTGGGTCG      800

TTGCCCCTCCTCTCTCCTTTACTCCTTTCCCATTTAATGGTCACGAGACCGTCTCCCCCAAATTTCTCACTTCCCCTAATTTCCTCTCGCGTTTATTGTT      900

CCTAATTCGAAAATTCCCATTTCTCAAGGTACGGTTCCGATCTTCGTCTGCTTCATACTCTCGTTTTCTCGTTTCTCGCGTTTATTGTT      1000

GATATGTTGTTGATCTTCGCGTTTTGGTCATCACTTCAAGATCTGTAGGATTCAAAGTTCGTTCTATCGATTTGGATAGATCTAGCAGGTGAAATCGTT      1100

TGTCAAAGTTTTTATTCTTTGGGTGCGTGGATTAATTTTGTTAGAGATGATTGTTATCGTTGCTTGAATGCAAGATTGTTGCAGCATTACTGAGTTC      1200

TATTTCGTGCTCTTTGTTTATTGCTGCTTGATATTTCTTGTTGAAAACAGGTTTTAAAAGATATTGCAAAGATGTCTTCTGATTCGTCCCGGGA      1300
                                                                                                  -1
                                                                                                  MetSerSerAspSerSerArgGl
AGAGAATGTGTACTTGGCCAAGTTACGCGACGCAAGCTGAGCGTTACGAGCAAATGGTTGATTCATGGAGAAAGTTCACAAAGACCTGAGAGCAGGAGA      1400
uGluAsnValTyrLeuAlaLysLeuArgAlaGluGlnAlaGluArgTyrGluGlnMetValGlnPheMetGluLysValAlaLysAspLeuArgAlaGlyGlu      1500
CTTACTGTTGAAGAGAGAATCTCTGTCTGTTACTACAAGAACGTGATTGGTGCTAGAGAGCTTCTTCGGAGGATTATCTCTTCATTGAGCAGAAGG
LeuThrValGluGluArgAsnLeuLeuSerValAlaTyrLysAsnValIleGlyAlaArgArgAlaSerTrpArgIleIleSerSerIleGluGlnLysEg      1600
AAGATAACGCAGGGCACAGTGATCATGTTTCGATTATCAAGGATTACAGAGGCAAGATTGAAACTGAGCAAGATTTGTGATGGCATTTTGAACCT
luAspAsnArgGlyArgSerAspHisValSerIleIleLysAspTyrArgGlyLysIleGluThrGluLeuSerLysIleCysAspGlyIleLeuAsnLe      1700
TCTTGAGGCTCATCTCATTCCTTCTCTGCTTCTCCAAAGTTTTTTACCTGAAGATGAAGGAGATTACATCGGTGCCTTGCTGAATTCAAG
uLeuGluAlaHisLeuIleProIleAlaIleSerLeuAlaGluSerLysValPheTyrLeuLysMetLysGlyAspTyrHisAspTyrLeuAlaGluVal      1800
ACTGGTCCTGAGAGAAAGAAAGCTGCTGAGAGCACTCTTGTTGCCTACAAGTTCGCTCAAGGTTATCATCTTGTTTCTCTTCTAACCATATAGAGTGCT
ThrGlyAlaGluArgLysGluLysAlaAlaGluSerThrLeuValIleAlaTyrLysSerAlaGln                                      1900
                                                                                                  AspIleAlaLeuAlaAspLeuAlaProThrHisProIleArgLeuGlyLeuAl
TCTTAACTTCTCTGTTTTCTACTATGAGATTCTCAACTCATCTGATCGTCCGTTGAGTCTCGCAAAGCAGTCTGTGTTACTTTTCATTACGTATGC      2000
eLeuAsnPheSerValPheTyrTyrGluIleLeuAsnSerSerAspArgAlaCysSerLeuAlaLysGln
TTTCTGTCATTAACTAACGAAACCTATATTCCAACTGTTTTGCTTGATTAAACTTAGCACATAAGAGGTTCTTTTTTCTATCTATCGTTCATAATGTGCC      2100

AAAAACGTTGTAGGTAGTTAACTTGTTACACTTCCATGTGGGTAGACTTTCCATATGTTCTAGTAATGGTTGTGATAGATTGCTTACCATAGCCTGACCGAT      2200

AGGGTTGATGTAATTTTGATGATGGGCAGAATTGGGAAACTAGATCCTGTGAATGGTTCCATACACTAGTGAAATTTGGACAACCTTCACATTCGGATT      2300

CTAGTTTGATTCAAGTACCTTTTGTTTAGTTACTAATATGATTTTTCATGGTGGTAGGCTTTTGATGAGGCAACTCCGGAGCTAGACACATTGGGAGAGG      2400
                                                                                                  AlaPheAspGluAlaIleSerGluLeuAspThrIleGlyGluG
AATCATACAAAGGACAGTACATTGATCATGCAGCTTCTCCGTGACAATCTCACCCTCTGKACTCTCTGACCTCAAGTGACTTGTCCTCTTTACACTCATCA      2500
luSerTyrLysAspSerThrLeuIleMetGlnLeuLeuArgAspAsnLeuThrLeuTrpThrSerAspLeuAsn
TTGTTACGATATCTTAGTGATTGACATCATGCAGTTAAGATTCTTTGTTTTCCGCATAACATTTGTGTCAATAATCTAATCTCCAGGACGAAGCTGGT      2600
                                                                                                  AspGluAlaGly
GATGATATCAAGGAAGCCCCGAAAGAGGTGCAGAAAGTTGATGAACAAGCCCAACCACCACCTTCGCAGTGATAAAATCAGATCCATGGAATGATTGCA      2700
AspAspIleLysGluAlaProLysGluValGlnLysValAspGluGlnAlaGlnProProProSerGln...
GACAAAAAGATATATGGCTTGTTGTTTTTAAACAGAAAAAAACTTGTAGTTTCCTTAAACATGGGCTGTAGTTTCCTTAAACATGGATTTGTAG      2800

TAGTAATTGGACTGCATGATTTGGTTATCGATGGTTAAAACTCTTGTGTGCTTCGCACATGTTTTGGGTTGTTGCGTTGTCGGTTCACTATTAG      2900

ACTGCATTGCATTACATGGTCCGGTCTAAAAGTTGAAAACTTGCTTCCTTCTCTGATTGGGGTTTAGTTGTCTTTTAGATTATGCTTCTCTAGGTGGAAT      3000

GTTCCCCAAAGTGAATCGGTTAAGACTCAAGAGAGTTGTTCAATGAAACGAAATAGAAATGGAATTTCACAACTTTAAACGAAAGATAAAGCCTCTTTCG      3100

GTTTGGAACAACTGTTAGGTAACACATATAATGGAAATTACAAATCATAAATCCTCATGACAATAAGCTAAAGGTTGGATTAATTAATAAATGACATGT      3200

GAATGGGAGGTAACATTAACTGGTCCAAAATAAATAATTAATGTAATGACACATTCACATAATAATATATCTCGAATTGAAATCCCACCGAGAGTGCCTGC      3300

TTTTCCACTGAGTTCTCTCATATATATCTCTTNCTCTCTATACGGAATTGTATCTCTCACTTTTGTTTCAATGGCTTCAGCTACTTCCGTTCATAGCCTG      3400

GTCATTTCTTCTTTCTATTCTACCTCTTTGTTTTCTCCATTACTGTTTCTGACTTGTCGTTCCTTCGTTTGATTTTTTGGCACAGGGGTTCTGGA      3500

GAAGATGGGCAACTAGGACTTGGAACCGATGAAGAAAAGGAATTGGCTTCCGTCGTCGACGCTCTTGAGCCTTTCAACGTTCGCTCCGTCGTCGGTGTA      3600

GCCGCAATTCCCTCGCCATTTGCGATGATGGCAAGGTTACTTATCCAATTTCCTTTATGTGTCGTTGTTGTCTTCTTTGGTTGGTTTCGCTTAGAACT      3700

AACTAAAGTACAATGCTTTGCTTTGATTTTGCGATTTGATTCTAAGCCATGTCTTAAGATTTTCTATGTTCGCTTTACATGGTTTTTGAGATCTGTA      3800

GGACCAATTAAAAAGGGTTTAAAGCTCGTATTTTTTTCTTTGTCCTGAAGTCCAACTAAAATGCAACCAATATGTTTGTCTGAGGTTACTCTCGAATAGA      3900

TCATCAGACTCTGGTTCTTGTCTCCATGGTGTAGTTTATGATTGATGTGTCACCAACATATAGAAATAATATGGTCTCTTAAATGGTTTCTCAGCTGTTT      4000

ACATGGGGTTGGAATCAAAGGGGTACATTAGGACACCCACCTGAGACAAAAACAGAAAGCACCCAAGTCTAGTTAAGTCTCTTGCCAACGTCAAGATTG      4100

TTCAGGTAAAACTCTTACAAATTATTTAAGTATTTTTGGCTCTAATGCTGTTTCTCGTTGTGATTTACTCTGAATTGCCGTTTCTTGTAATTGATCATAG      4200

GCAGCTATTGGTGGCTGGCATTGTTTAGCTGTCGATGATCAAGGCCGAGCATATGCTTGGGGTATGCATTTTCTCTCCTCAGCTTCTTTCTAATGATTGTG      4300

ACCTCAATGATCTTAATGATCTTAAGTTGTTCACCTTTTTGTTATATAATCTACTTAGGTGG                                          4362
```

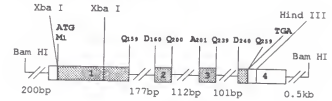
Figure 3-4. Physical structure of the GF14 genes.

Restriction map of the insert of each genomic clone is shown at the top. Coding regions are shown by hatched blocks, and 5' and 3' untranslated regions are shown by open blocks. The 5' and 3' flanking sequences and introns are designated by lines. Numbers above the gene structure indicate the codon positions, and the numbers below show the size of the introns. The amino acid split by introns are labeled. Start and stop codons are also indicated.

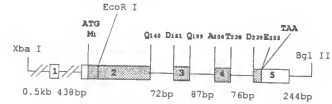
GRF1-GF14 chi Genomic



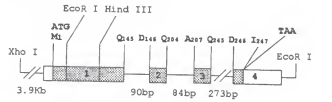
GRF2-GF14 omega Genomic



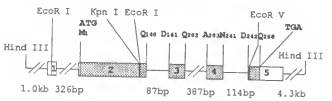
GRF3-GF14 psi Genomic



GRF4-GF14 phi Genomic



GRF5-GF14 upsilon Genomic



GRF2-GF14 psi and GRF5-GF14 upsilon each display an extra intron in the 5' untranslated leader of their cDNA sequences in addition to the three introns in the same conserved positions as those of GF14 omega, chi, and phi (Fig. 3-4). The positions of introns within the protein coding sequences are indicated in Figure 3-2. It is noteworthy that the three conserved intron positions are also present in the two GF14 genomic sequences of maize, a monocot (de Vetten and Ferl, 1994). However, unlike those in maize GF14 genes, the introns of the Arabidopsis GF14 genes are not conserved either in length or nucleotide sequences (Fig. 3-4). In mammals, one available genomic sequence of human

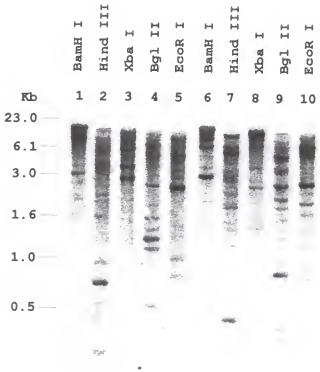
14-3-3 eta displays a very divergent genomic structure from those in plants. It contains only one intron of approximate 8 kb that is located at the 5' end of the cDNA sequence (Muratake et al., 1996).

Southern Blot Analyses of the Arabidopsis 14-3-3 Multigene Family

To approach the size and divergence of 14-3-3 multigene family in Arabidopsis, Southern analysis was performed under low-stringency conditions using probes made from two of the most distantly related genes, GF14 phi and GF14 epsilon. The Arabidopsis genomic DNA restricted with five different restriction enzymes produced 7 to 15 bands that hybridized with the GF14 phi probe (Fig. 3-5, lane 1-5). Similar results were obtained using a probe made from divergent GF14 epsilon cDNA clone under the same conditions (Fig. 3-5, lane 6-10). Many bands hybridizing to the epsilon probe are also recognized by the phi probe, indicating that these divergent probes cross hybridize at this low stringency.

Figure 3-5. Genomic Southern analyses reveals a large GF14 gene family.

Arabidopsis genomic DNA (1.2 microgram) was restricted with BamH I, Hind III, Xba I, Bgl II, and EcoR I, respectively, resolved on a 0.8% agarose gel, blotted to nylon membrane, and hybridized with a probe made from GF14 phi (lane 1-5) under the conditions as described in Materials and Methods. After exposure the same blot was stripped and hybridized with a probe made from GF14 epsilon (lane 6-10). Kb is indicated by numbers on the left.


BamH I
Hind III
Xba I
Bgl II
EcoR I
BamH I
Hind III
Xba I
Bgl II
EcoR I
Kb
1 2 3 4 5 6 7 8 9 10
23.0
6.1
3.0
1.6
1.0
0.5

Evolution of 14-3-3 Sequences

An Ancient Split among Plant 14-3-3s

To study the evolution of 14-3-3 proteins, protein phylogenetic trees were initially generated based on the alignment of truncated Arabidopsis GF14 protein sequences in which highly variable parts of N- and C-termini were removed (Fig. 3-2). These regions are so divergent that proper alignment becomes problematic (Ferl et al., 1994). Similarly truncated Entamoeba 14-3-3-1 protein sequence was also included in the alignment (data not shown) and later employed as an outgroup because *Entamoeba histolytica*, an amitochondrial protozoan, represents the most divergent species in term of systematic classification.

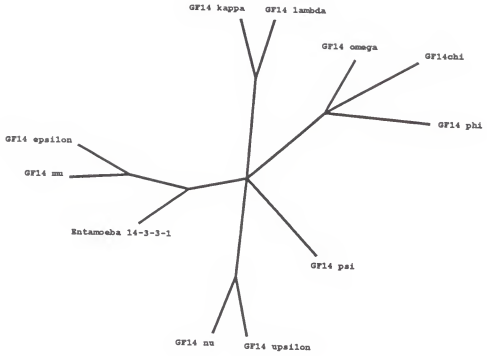
As shown in Figure 3-6A and B, unrooted networks were made using both neighbor-joining and protein parsimony methods from PHYLIP and only those branches that survived the bootstrap analysis at 80% are indicated. Both trees place GF14 epsilon and mu in one cluster, GF14 kappa and lambda in one cluster, and GF14 psi by itself. GF14 omega, GF14 chi, and GF14 phi are also grouped in one cluster. The placement of GF14 upsilon and GF14 nu is the only variation in the two networks. Neighbor-joining (Fig. 3-6A) supports the grouping of GF14 upsilon and nu with a subtending branch, whereas parsimony (Fig. 3-6B) leaves upsilon and nu as independent basal branches. The grouping of GF14 omega, chi and phi is also supported by the fact that this group lacks the 5' intron characteristic of GF14 psi and upsilon.

To further characterize the evolutionary relationship of 14-3-3s among different species, especially in plants, all available 14-3-3 protein sequences from plants and certain representative sequences from other organisms were retrieved from protein

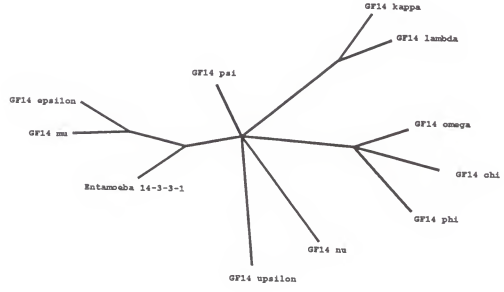
Figure 3-6. Unrooted neighbor-joining (A) and protein parsimony (B) network analysis of Arabidopsis GF14 proteins and Entamoeba 14-3-3-1.

The branch lengths shown in the network are proportional to the branch lengths calculated by neighbor-joining and protein parsimony programs contained in PHYLIP program package (version 3.5). Only those branches that survived bootstrap analysis at 80% are indicated.

A


GF14 kappa
GF14 lambda
GF14 omega
GF14chi
GF14 phi
GF14 epsilon
GF14 mu
Entamoeba 14-3-3-1
GF14 psi
GF14 nu
GF14 upsilon

B


GF14 kappa
GF14 lambda
GF14 psi
GF14 epsilon
GF14 mu
GF14 omega
GF14 chi
Entamoeba 14-3-3-1
GF14 phi
GF14 nu
GF14 upsilon

database of GenBank (Table 3-3). They were truncated to eliminate the highly divergent N- and C-termini, aligned (Fig. 3-7), and trees were generated in a similar manner as described (see above). As shown in Figure 3-8, both trees show similar but nonidentical topology, indicating that the major evolutionary groupings are correct, even though the relative branch positions of some ancient branches remain uncertain.

Most plant sequences (subgroup III) cluster separately from those of mammals (subgroup I) with a high degree of confidence in both trees, indicating that mammalian and plant 14-3-3 lineages have been on distinct and separate evolutionary tracts since their earliest divergence. This result is also confirmed by the significant divergence in genomic structure of plant and mammalian 14-3-3 genes. Similar conclusions were also reached by previous studies that were based on fewer sequences (Ferl et al., 1994; Wang and Shakes, 1996).

The notable exceptions are the 14-3-3 isoforms indicated as subgroup II in Figure 3-8. This distinct group includes 14-3-3 proteins from both plants and mammals and the grouping is supported in both trees. The plant isoforms in this group include Arabidopsis GF14 epsilon, mu, Tobacco CZ1, and *V. faba* 14-3-3b and they form a distinct grouping within the phylogenetic tree of plant 14-3-3 proteins. Arabidopsis GF14 epsilon and GF14 mu are more similar to tobacco CZ1 and *V. faba* 14-3-3b, respectively, than to other Arabidopsis homologs. The separation of these isoforms away from Subgroup I and II is clearly supported by very strong bootstrap values (Fig. 3-8). Early branches in Subgroup II are weakly supported by bootstrap analysis, indicating ancient branches from an as yet unresolved basal polychotomy.

At the amino acid sequence level, GF14 epsilon and mu represent the most divergent ones among all Arabidopsis isoforms and they are more identical to each other

Table 3-3. 14-3-3 sequences in this study

Organism	Gene type	Accession No.	Reference
Plant			
Arabidopsis thaliana	GF14 Omega (ω)	M96855	(Lu et al., 1992)
Arabidopsis thaliana	GF14 Chi (χ)	L09112	(Lu et al., 1994c)
Arabidopsis thaliana	GF14 Phi (ϕ)	L09111	(Lu et al., 1994c)
Arabidopsis thaliana	GF14 Psi (ψ)	L09110	(Lu et al., 1994c)
Arabidopsis thaliana	GF14 Upsilon (υ)	L09109	(Lu et al., 1994c)
Arabidopsis thaliana	GF14 Epsilon (ε)	U36446	This report
Arabidopsis thaliana	GF14 Kappa (κ)	U36447	This report
Arabidopsis thaliana	GF14 Lambda (λ)	U68545	This report
Arabidopsis thaliana	GF14 Mu (μ)	U60444	This report
Arabidopsis thaliana	GF14 Nu (ν)	U60445	This report
Hordeum vulgare (Barley)	Hv14-3-3a	X62388	(Brandt et al., 1992)
Hordeum vulgare	Hv 14-3-3b	X93170	(Brandt et al., 1992)
Nicotiana tabacum (Tobacco)	CZ1	S76737	(Chen et al., 1994)
Oenothera hookeri		X62838	(Hirsch et al., 1992)
Oryza sativa (Rice)	S94	D16140	(Kadou et al., 1993)
Oryza sativa	GF14-b	U65956	Schultz et al., unpublished
Oryza sativa	GF14-c	U65957	Schultz et al., unpublished
Oryza sativa	GF14-d	U65958	Schultz et al., unpublished
Pisum sativum (Pea)		U15036	(Stankovic et al., 1995)
Lycopersicon esculentum(Tomato)	GF14-T3	L29151	(Laughner et al., 1994)
Lycopersicon esculentum	GF14-T4	L29150	(Laughner et al., 1994)
Solanum tuberosum (Potato)		X97724	Wilczynski and Szopa, unpublished
Solanum tuberosum (Potato root)		X87370	Wilczynski and Szopa, unpublished
Vicia faba	Vfa-1433a	Z48504	Saalbach et al., unpublished
Vicia faba	Vfa-1433b	Z48505	Saalbach et al., unpublished
Zea mays (Maize)	GF14-12	M96856	de Vetten et al., 1992
Yeast			
Saccharomyces cerevisiae	BMH1	X66206	(van Heusden et al., 1992)
Saccharomyces cerevisiae	BMH2	x84817	(van Heusden et al., 1995)
Mammal			
Homo sapiens (Human)	epsilon	U54778	(Chong et al., 1996)
Rattus norvegicus (Rat)	beta	S55223	(Watanabe et al., 1993)
Rattus norvegicus	epsilon	M84416	(Roseboom et al., 1994)
Rattus norvegicus	eta	D17445	(Watanabe et al., 1991)
Rattus norvegicus	gamma	S55305	(Watanabe et al., 1993)
Amoeba			
Entamoeba histolytica	14-3-3-1	U13418	Samuelson et al., unpublished

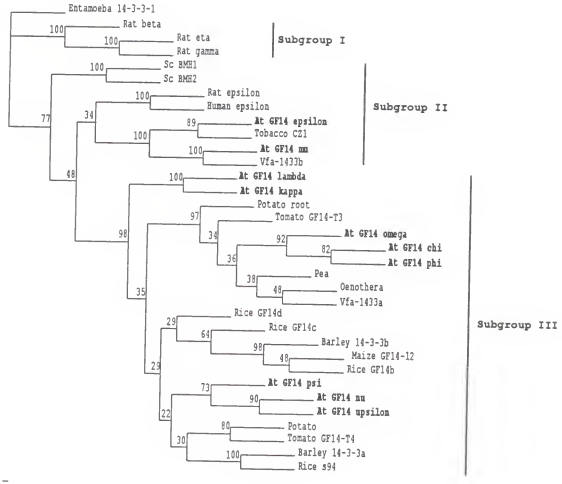
Figure 3-7. Alignment of truncated 14-3-3 homolog sequences.

The sequences derived from those listed in Table 3-3 were truncated at conserved residues in order to facilitate an unambiguous alignment.

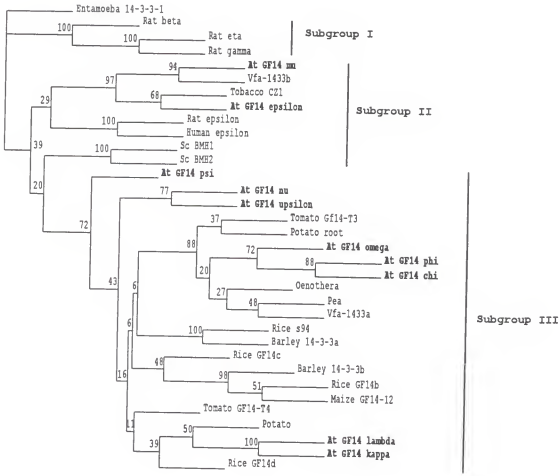
```
Rat eta          MKAV------TKLWHFLSNEVDRNLSFVAYNVVVGARSSWAVISSIFSQKTMADGNEKK-LEKVEAYEKEIKELETVCNDVLALLDKFLIKNCNDFQTESKVVFYLKMKGD  103
Rat gamma        MKNV------TKLWHFLSNEKNRLLSFVAYNVVVGARSSWAVISSIFSQKTSADGNEKK-IDMVEAYREEIKELEAVCQDVLSLLDNYLIKNCSETQYESKVVFYLKMKGD  103
Rat beta         MKAV------TEQGHELSNEKNRLLSFVAYNVVVGARASSWAVISSIFSQKTEAN--EKK-QDMGKEYREKIEAELQDICSDVLELLDKYLIANATNA--ESKVVFYLKMKGD  103
At GI14 lambda   MKQL---VTGATFAEELTVEERNLLGVAYKNVIGSRIAAWRIVSSIEQKEESKQNEDN-VELVKDYRKKVEEELSSVCSGILKLLDEHLIPSAGAS--ESKVVFLKMKGD  104
At GI14 beta     MKQL---VSGATFAEELTVEERNLLGAYKNVVGSRIAAWRIISSIEQKEESKQNEDN-VELVKDYRAKVEEELSSVCSGILKLLDEHLIPSAGAS--ESKVVFLKMKGD  104
Potato           MEKV------AAVDVEELTVEERNLLGVAYKNVIGARAAWRIISSIEQKEESKQNEDN-VSSIKDYRVKIEAELSKICDGILSLLESKHLVPSASTA--ESKVVFLKMKGD  104
At GI14 mu       MKSVAQL-----HV-DLTVEERNLLGVGYKNVIGERAAWRIISSIQKEAVNDNVN-VKSIKEYMEKVELELSNICIDIHSFVLDEHLIPSASEG--ESTVFNHMKGD  101
Vfa-1433b        MKNVAHL-----DV-ELTIEERNLLGVGYKNVIGARAAWRLISSIEQKEEGNDVN-AKSIKEYRQKVEKELSKICHDVFSLLDSHFIASHAD--ESTVFYLKMKGD  103
Tobacco CS1      MKTVANR-----DV-ELTVEERNLLVVGYKNVIGARRASWRLISSIEQKEESKGHDQH-VKSIKTYQQKVEDELITVCNLL-TLDVIDEHVVPESTSG--ESTVFYTKMKGD  100
At GI14 epsilon  MKSVAQL-----DV-ELTVEERNLLVSGYKNVIGARAAWRLISSIEQKEENKGGEDK-VKSLKNYRQKVEEELKLICCDIIDVLDNHLIPSANTQ--ESKVFYTHMKGD  101
Rat eps          MKSVAQM-----DV-ELTVEERNLLGAYKNVVGARAASWRLISSIEQKEENKGGEDK-LAMIAEYRQMVETELKLICCDIIDVLDNHLIPSANTQ--ESKVFYTHMKGD  101
human epsilon    MKSVAQM-----DV-ELTVEERNLLGAYKNVVGARAASWRLISSIEQKEENKGGEDK-LAMIAEYRQMVETELKLICCDIIDVLDNHLIPSANTQ--ESKVFYTHMKGD  101
Pea              MEKVSNAA----DSEELTVEERNLLGVAYKNVIGSRRASWRLISSIEQKEESRQNEDN-VAVIADYRSKIEEELSSVCDGILKLLDTKLIPSASSG--DSKVFYLKMKGD  103
Vfa-1433a        MEKVTAAV----ESEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRQNEDH-VTSIADYRSKIEEELSSVCDGILKLLDERLIPSAAGG--DSKVFYLKMKGD  103
At GI14 omega    MEKVSAAV----DGDELTVEERNLLGVAYKNVIGARRASWRLISSIEQKEESRQNDN-VTSIKEYRSKIEEELSGICDGILKLLDSRLIPSAAGG--DSKVFYLKMKGD  103
oenothera        MEKVCAAA----DSEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRQNDN-VTSIKEYRSKIEEELSGICDGILKLLDSRLIPSAAGG--DSKVFYLKMKGD  103
At GI14 phi      MEKVADAV----DKDELTVEERNLLGVAYKNVIGARRASWRLISSIEQKEESRQNDN-VTTIADYRSKIETELSNICDGILKLLDERLIPSAAGG--DSKVFYLKMKGD  103
At GI14 chi      MEKVAAAV----DKDELTVEERNLLGVAYKNVIGARRASWRLISSIEQKEESRQNDN-VTTIADYRSKIETELSNICDGILKLLDTKLVPAGANG--DSKVFYLKMKGD  103
Tomato GI14-T3   MEKVNSLS----GSEELTVEERNLLGVAYKNVIGARAAWRLISSIVQKEESRQNEDH-VNSIAEYRSKIEMELSKICDGILKLLDEKLIPSATSG--DSKVFYLKMKGD  103
Potato root      MEKVVAAL----DY-ELTVEERNLLGVAYKNVIGARRASWRLISSIEQKEESRQNEDH-VASIKKYRSQIEMELTSICNGILKLLDEKLTGSAATG--DSKVFYLKMKGD  103
Barley 14-3-3a   MEKVAATA----DVSELTVEERNLLGVAYKNVIGARRASWRLISSIEQKEESRQNEDH-VTTIKDYRGKIETELTKICDGILKLLESHLVPSSTAP--ESKVFYLKMKGD  103
Rice s94         MEKVAMTT----DVGELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESKQNEAY-VASIKEYRTRIETELSKICNGILKLLDNHLVPSATAA--ESKVFYLKMKGD  103
Tomato GF14-T4   MEKVAMTA----DVEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRQNEDH-VMTIKEYRSKIEADLSKICDGILKLLDSHLVPTSTAS--ESKVFYLKMKGD  103
Rice GF14d       MEKVAAAAGGAGSGGELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEEGRGNDAH-AATIRSYRGKIEAELAAICDGILALLDSHLVPSAGAA--ESKVFYLKMKGD  107
At GF14 nu       MEKVANTV----DTDELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRGNDN-VEIIKDYRGKIETELSKICDGILNLLDSHLVPTASLA--ESKVFYLKMKGD  103
At GF14 upsilon  MEKVAKTV----ETEELTVEERNLLGVAYKNVIGARAAWRLISSIEQSDSRGNDEN-VEIIKDYRGKIETELSKICDGILNLLESHLVPTASLA--ESKVFYLKMKGD  103
At GF14 psi      MEKVAKTV----DVEELSVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRGNDEN-VEIIKDYRGKIETELSKICDGILNLLEAHLIPAASLA--ESKVFYLKMKGD  103
Barley 14-3-3b   MEKVANTV----DSEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRGNEDR-VTLIKDYRGKIETELTKICDGILKLLESHLVPSSTAP--ESKVFYLMKGD  103
Rice GF14c       MEKVANTV----DVEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRGNEDR-VTLIKDYRGKIEVELTKICDGILKLLESHLVPSSTAP--ESKVFYLMKGD  103
Maize GF14-12    MEKVANTV----DSEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEESRGNEDR-VTLIKDYRGKIETELTKTCDGILKLLESHLVPSSTAP--ESKVFYLMKGD  103
Rice GF14c       MEKVAKTV----DVEELTVEERNLLGVAYKNVIGARAAWRLISSIEQKEEGRGNEDH-VTLIKEYRGKIETELTKICDGILKLLESHLVPSSTAP--ESKVFYLMKGD  103
Sc 8862          MKTVAS------SGQELSVEERNLLSVAYKVVIGARRASWRIVSSIEQKEESKESHQVELISCYRSKIETELTKICTDILSLLDSHLVPSSTAA--ESKVFYMKGD  102
Sc 8862          MKAVAS------SGQELSVEERNLLSVAYKVVIGARRASWRIVSSIEQKEESKKSHQVELISYRSKIETELTKICSDILSVLDSHLITSATTG--ECKVFYYMKGD  102
Entamoeba 14-3-3-1  MKQVAEN----EA-ELSIEENNLLSVAYKNVIGARRASWRIISSLQKEEAQKGNDH-VEIIKGYKAKIEKELSTCCDDVLSVTQEHLLPKASTS--ESKVFYIKMEGD  101

Rat eta          YYAYLAEVASGDKKN-SVVEASEAAYKEAFIISKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKQAFDDAIAELDTLNEESYKDSTLIMQLLRDNLTL  205
Rat gamma        YYAYLAEVATGEKRA-TVVESSEKAYSEALNKISKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKQAFEDAIAELDTLNEDSYKDSTLIMQLLRDNLTL  205
Rat beta         YFAYLSEVASGDNKQ-TTVSNSQQAYQEAFKISKEMQPTHPIRLGLALNFSVFYYEILNSPEKACSLAKTAFDEAIAELDTLNEESYKDSTLIMQLLRDNLTL  206
At GI14 lambda   THATNMAETKGD-EKKTAADTMLAYKAAQDIAAADLAPTHPIRLGLALNFSVFYYEILNSSDKACNMAKQAFEEAIAELDTLGEESYKDSTLIMQLLRDNLTL  206
At GI14 beta     YHATLAEFKGD-EKNTAADTAMIAYKAAQDVAIDALAPTHPIRLGLALNFSVFYYEILNSSEKACSMAKQAFEEAIAELDTLGEESYKDSTLIMQLLRDNLTL  206
Potato           YHATLAEFTGSAEKAAADTAMIAYKAAQDIAVAELAPTHPIRLGLALNFSVFYYEILNSPDRACSLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  206
At GI14 mu       TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDDAIAELDTLGEESYKDSTLIMQLLRDNLTL  206
Vfa-1433b        TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  206
Tobacco CS1      TYATLAEFKGDDREKAA-DQSLAAYEAATATAEADLAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNFT  202
At GI14 epsilon  TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDSLNEESYKDSTLIMQLLRDNLTL  203
Pea              TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLSEESYKDSTLIMQLLRDNLTL  203
Vfa-1433a        TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
At GI14 omega    TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
Oenothera        TYATLAEFKGDKEAAADTAMIAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
At GI14 phi      TYATLAEFKGDKEAAADTAMIAYKAAQDIANKLAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
At GF14 chi      TYATLAEFKGDKEAAADTAMIAYKAAQDIANKLAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
Tomato GF14-T3   TYATLAEFKGAKEKEAA-EMTLAYKAAQDIAAELAPTHPIRLGLALNFSVFYYEILNSPERACNLAKQAFEEAISELDTLSEESYKDSTLIMQLLRDNLTL  205
Potato root      TYATLAEFKTGTEKKEAA-EMTLSAYKSAQDIANGKLAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
Barley 14-3-3a   YHATLAEFKGAKEKKEAA-EMTLVAYKSAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDDAIAELDTLNEESYKDSTLIMQLLRDNLTL  204
Rice s94         TYATLAEFKGAKEKKEAA-EMTLVAYKSAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAIAELDTLGEESYKDSTLIMQLLRDNLTL  205
Tomato GF14-T4   TYATLAEFKTGTEKKEAA-EMTLAYKSAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLSEESYKDSTLIMQLLRDNLTL  205
Rice GF14d       TYATLAEFKSGDEKKQAA-EMTWAYKAAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  209
At GF14 nu       TYATLAEFKTGAEKKEAA-ESTLVAYKSAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  205
At GF14 upsilon  TYATLAEFKTGAEKKEAA-ESTLVAYKSAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDRACSLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  205
At GF14 psi      TYATLAEFKAGAEKKEAA-ESTLVAYKSADDIATAELAPTHPIRLGLALNFSVFYYEILNSPDRACSLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  205
Barley 14-3-3b   TYATLAEFKSGTEKKDAA-EMTWAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLSEESYKDSTLIMQLLRDNLTL  205
Rice GF14b       TYATLAEFKTGAEKKDAA-EMTWAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLSEESYKDSTLIMQLLRDNLTL  205
Maize GF14-12    TYATLAEFKTGAEKKDAA-EMTWAYKAAQDIALAELAPTHPIRLGLALNFSVFYYEILNSPDRACNLAKQAFDEAISELDTLSEESYKDSTLIMQLLRDNLTL  205
Rice GF14c       TYATLAEFKTGAEKKEAA-ESTWAYKAAQDIALADLAPTHPIRLGLALNFSVFYYEILNSPDKACNLAKQAFDEAISELDTLGEESYKDSTLIMQLLRDNLTL  205
Sc 8862          TYATLAEFSSGQAAKEAT-NASLEAYKTAEEIATTLEPTHPIRLGLALNFSVFYYEIQNSPDKACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTL  204
Sc 8862          TYATLAEFSSGDAAKEAT-NSSLEAYKTAEEIATTLEPTHPIRLGLALNFSVFYYEIQNSPDKACHLAKQAFDDAIAELDTLSEESYKDSTLIMQLLRDNLTV  204
Entamoeba 14-3-3-1  YYAYEAETVTDEKKKEVA-DKSLAAYTEATKISNAELAPTHPIRLGLALNFSVFYYEIMNDADKACQLAKQAFDDAIADLDVEGMEMYKDSTLIMQLLRDNLTL  203
```

Figure 3-8. Bootstrap majority-rule consensus trees derived from 100 neighbor-joining (A) and protein parsimony (B) replicates with alignment shown in Fig. 5.

A


Entamoeba 14-3-3-1
Rat beta
Rat eta
Rat gamma
Sc BMH1
Sc BMH2
Rat epsilon
Human epsilon
At GF14 epsilon
Tobacco C21
At GF14 nu
Vfa-1433b
At GF14 lambda
At GF14 kappa
Potato root
Tomato GF14-T3
At GF14 omega
At GF14 chi
At GF14 phi
Pea
Oenothera
Vfa-1433a
Rice GF14d
Rice GF14c
Barley 14-3-3b
Maize GF14-12
Rice GF14b
At GF14 psi
At GF14 nu
At GF14 upsilon
Potato
Tomato GF14-T4
Barley 14-3-3a
Rice s94
Subgroup I
Subgroup II
Subgroup III

B


Entamoeba 14-3-3-1
Rat beta
Rat eta
Rat gamma
Subgroup I
At GF14 nu
Vfa-1433b
Tobacco CZ1
At GF14 epsilon
Rat epsilon
Human epsilon
Sc BMH1
Sc BMH2
Subgroup II
At GF14 psi
At GF14 nu
At GF14 upsilon
Tomato Gf14-T3
Potato root
At GF14 omega
At GF14 phi
At GF14 chi
Oenothera
Pea
Vfa-1433a
Rice s94
Barley 14-3-3a
Rice GF14c
Barley 14-3-3b
Rice GF14b
Maize GF14-12
Tomato GF14-T4
Potato
At GF14 lambda
At GF14 kappa
Rice GF14d
Subgroup III

than to other homologs (Fig. 3-1, Table 3-2). In addition, *E. coli* expressed GF14 epsilon and mu displayed the weakest immunoreactivity with the monoclonal antibody relative to all other isoforms (data not shown). These data suggest that GF14 epsilon, mu, tobacco CZ1, and *V. faba* 14-3-3b all belong to a novel class of genes within the plant 14-3-3s, distinct from the class that contains all other plant sequences. The duplication event that led to the formation of these two ancient classes of plant 14-3-3 isoforms apparently predated the divergence of Arabidopsis, tobacco, and *V. faba*.

Plant 14-3-3s versus those of Mammals

The tight clustering of nonepsilon plant 14-3-3 isoforms (subgroup III) in both trees indicates that most of the present-day isoform diversity arose after the separation of epsilon-like and nonepsilon isoforms through independent duplications within each subbranch. As shown in both trees, the nonepsilon isoforms from monocots appear to separate from those of dicots (Fig. 3-8). The branching patterns of some nonepsilon isoforms, such as these in Arabidopsis, indicate that recent duplication events were responsible for the generation of some isoforms, in contrast to the situation in mammals in which various isoform groups were well established before the point of mammalian and possibly vertebrate divergence (Wang and Shakes, 1996).

Discussion

Arabidopsis Contains a Complex 14-3-3 Gene Family

The ten Arabidopsis 14-3-3 genes identified and characterized herein represent the largest 14-3-3 gene family in any species examined to date. An exhaustive search of the latest Arabidopsis dbEST (with over 25,000 entries) using all ten 14-3-3 sequences retrieved only EST sequences that were identical to the probes (data not shown). It is highly likely that these ten distinct 14-3-3 sequences represent all members of 14-3-3 protein family in Arabidopsis. Initial results from northern analyses of Arabidopsis suspension cells (data not shown) and the presence of all ten unique sequences in the dbEST indicate that they are all expressed in Arabidopsis at the mRNA level.

In spite of their high conservation of amino acid sequences, different Arabidopsis 14-3-3 isoforms also maintain their distinctiveness especially at their N- and C-terminal ends (Fig. 3-2), suggesting they may encode for different functions.

Evolution of Plant 14-3-3s

Plant 14-3-3 proteins are divided into two main divergent classes, namely epsilon-like and nonepsilon isoforms. Arabidopsis GF14 epsilon, mu, tobacco CZ1, and *V. faba* 14-3-3b belong to epsilon-like isoforms whereas the rest of plant 14-3-3s belong to nonepsilon isoforms. It is still unclear whether the split that generated epsilon-like and nonepsilon isoforms occurred before the separation of monocots and dicots due to a lack of sequence data from monocot species. However, one intriguing observation is that

searching the dbEST of rice revealed that there is a family of 14-3-3 homologs similar to that of Arabidopsis. Thus it is very likely that there are Arabidopsis epsilon-like isoforms in rice as well as in other monocots. The deep branching pattern of plant epsilon-like isoforms from nonepsilon isoforms suggests that the split that produced these two lineages might have occurred during the very earliest divergence of ancient plant species (Fig. 3-8). The divergence between epsilon-like and nonepsilon isoforms was further demonstrated by the results from detailed analyses of DNA and protein sequences, dimerization domain mapping (Wu et al., 1997a), and immunoreactivity of expressed Arabidopsis 14-3-3 proteins (data not shown).

The evolutionary relationships among plant epsilon-like and mammalian epsilon isoforms remains to be fully investigated. Mammalian epsilon is the most unique isoform among all mammalian 14-3-3s (Ferl et al, 1994; Wang and Shakes, 1996). They are more similar to plant and yeast 14-3-3 isoforms than to the rest of mammals and they were thought to serve as a functionally conserved copy of the original ancestral 14-3-3 genes (Wang and Shakes, 1996). Based on the ancient divergence observed in current study, the Arabidopsis epsilon-like 14-3-3s may similarly serve ancient and essential functions different from those of the nonepsilon plant isoforms.

In Arabidopsis, some nonepsilon isoforms seem to arise from recent duplication events. It is still unclear how many nonepsilon isoforms were generated after the divergence of different species. Isolation and analysis of 14-3-3 proteins from additional plant species will provide a better picture of exactly when the latter duplication events occurred.

Plant 14-3-3 Functions

All phylogenetic analyses support early and clear divergence of plant nonepsilon and mammal lineages. Therefore, there are no evolutionary tracks that indicate specialized functions shared among the known mammalian and plant isoforms except perhaps the epsilon isoforms. Fundamental properties can be shared; but specialized functions cannot be shared by animal and plant isoforms as a result of evolutionary heritage. Evidence supporting this theory has been emerging since the early stages of plant 14-3-3 research. Specialized functions and activities that are as yet unique to plants include association with a DNA/protein complex (de Vetten et al., 1992; Lu et al., 1992), receptors for fusicoccin (Korthout and de Boer, 1994; Oecking et al., 1994), and binding and regulation of nitrate reductase (Bachmann et al., 1996a, 1996b; Moorhead et al., 1996). Fundamental properties and activities shared among animals and plants include phosphoprotein binding activities and participation in signaling pathways. The evidence for shared functions includes observations that an Arabidopsis 14-3-3 isoform can perform the functions of yeast and mammalian isoforms (Lu et al., 1994a), several plant isoforms are able to regulate the PKC activities like those from mammals (Chen et al., 1994; Lu et al., 1994a), and expressed Arabidopsis GF14 proteins bind to nitrate reductase (Bachmann et al., 1996a, 1996b; Moorhead et al., 1996) that contains a phosphoserine consensus sequence known to be bound by 14-3-3 proteins in mammals (Muslin et al., 1996). Therefore the next question about the diversity of 14-3-3 isoforms would be how different isoforms with similar fundamental properties carry out different or specific functions. One answer may be that different isoforms exhibit subtle quantitative or qualitative alterations in these

fundamental properties (Bachmann et al., 1996a) that result in the appearance of specificity.

Another answer may lie in the intrinsic characteristics of different 14-3-3 genes that determine their distinct expression patterns and their different ability to respond to differential external stimuli. The characterized 14-3-3 isoforms in Arabidopsis, GF14 chi, psi, omega, upsilon, and epsilon display certain degree of cell- and organ-specific expression patterns (Lu et al., 1992; Daugherty et al., 1996; Chapter 4). In addition, environmental stimuli influence 14-3-3 expression in plants as well. Some 14-3-3 isoforms were demonstrated to be induced under low temperature (Jarillo et al., 1994), induced by hypoxia (de Vetten and Ferl, 1995), down-regulated during acclimation to high salt (Chen et al., 1994), regulated by both salt and low temperature (Kidou et al., 1993), and induced after pathogen attack (Brandt et al., 1992). These observations suggest the involvement of specific 14-3-3s in specific signal transduction pathways, and distinct response profiles of various isoforms may simply serve to ensure expression of the fundamental activities during times of need.

The identification and initial characterization of the ten Arabidopsis 14-3-3 isoforms presents us with an unprecedented opportunity to fully understand the fundamental function(s) and diversity of 14-3-3 proteins within a single organism. Characterization of the biochemical properties of each isoform will lend further insight into their potential roles. Defining the phenotype of transgenic plants with antisense genes of different isoforms will contribute to the clarification of the pathways impacted by 14-3-3 members. Together with an appreciation of the localization profiles of all the isoforms,

we may garner greater understanding of the entire 14-3-3 gene family in Arabidopsis as well as those in other organisms.

CHAPTER 4
CHARACTERIZATION OF TRANSGENIC ARABIDOPSIS PLANTS OVEREXPRESSING ANTISENSE AND SENSE 14-3-3 GENES

Introduction

14-3-3 proteins are highly homologous, widely distributed, eukaryotic proteins. They have been indicated to be involved in a huge array of functions and activities in diverse organisms (for review, see Chapter 1). Most of the activities involving 14-3-3 proteins are signal transduction pathway related and 14-3-3 proteins appear to mainly exert their different effects in different organisms through interacting with other proteins.

The amino acid sequences of distinct 14-3-3 proteins are highly conserved except in the extreme N- and C-termini. 14-3-3 proteins from diverse species apparently share fundamental properties while they may still possesses certain degree of isoform specificity (Chapter 1).

Five Arabidopsis 14-3-3 proteins were identified as component(s) of G-box protein-binding complex and they were subsequently designated as GF14s (**G**-box **F**actor **14**-3-3 homologs) (Lu et al., 1992; Lu et al., 1994c). Subsequent studies identified another five new isoforms, making the total number of Arabidopsis 14-3-3 isoforms ten (Jarillo et al., 1994; Wu et al., 1997b). Based on their sequence similarity, Arabidopsis 14-3-3 isoforms can be grouped into two classes, epsilon-like and nonepsilon isoforms.

Epsilon-like isoforms contain two homologs, GF14 epsilon and mu. Sequence analysis indicated that epsilon-like isoforms are likely to play a different functional role than the rest of the isoforms (Wu et al., 1997b). In another study of the dimerization domain of Arabidopsis 14-3-3 chi, the N-terminal half region of chi (chi-NT) was demonstrated to be able to mediate dimerization with diverse isoforms (Wu et al., 1997a).

In addition to the participation in G-box protein-binding complex in Arabidopsis, plant 14-3-3 homologs were also found to be involved in other pathways. In spinach, homologs of GF14 proteins bind to and inhibit nitrate reductase (NR) (Bachmann et al., 1996a; Bachmann et al., 1996b; Moorhead et al., 1996) and these activities can be interchangeably carried out by Arabidopsis GF14 proteins *in vitro*, indicating that these functions are probably conserved in all plant species (Bachmann et al., 1996a; Bachmann et al., 1996b). GF14 homologs were also indicated to participate in perception of a phytotoxin, fusicoccin in several plant species (Korthout and de Boer, 1994; Marra et al., 1995; Oecking et al., 1994). Despite these discoveries, the exact functions of 14-3-3 proteins in Arabidopsis remain to unknown.

The reverse genetic approach, such as overexpression of sense or antisense genes in transgenic organisms, has been shown to be a powerful tool for the functional analysis of different plant gene products (Rodermel et al., 1988; Sonnewald et al., 1991; van der Krol et al., 1988; Weigel and Nilsson, 1995). In current studies, I established transgenic Arabidopsis plants in which antisense GF14 mu (anti-mu) was introduced to alter the message level of endogenous GF14 mu gene. I also generated transgenic Arabidopsis plants transformed with GF14 chi-NT to examine the potential changes caused by

overexpression of GF14 chi dimerization domain. Preliminary characterization failed to observe any phenotypic changes of these transgenic plants.

Materials and Methods

Subcloning and Sequencing

The cDNA encoding the Arabidopsis GF14 mu isoform was cloned in the antisense orientation into pBI121 vector (Clontech) between the 35S cauliflower mosaic virus promoter and NOS terminator (35S-anti-mu). In a similar manner, the cDNA encoding the first 139 amino acids of Arabidopsis GF14 chi isoform (chi-NT) (Wu et al., 1997a) was cloned into pBI121 (35S-chi-NT), replacing the beta-glucuronidase coding sequence. DNA sequencing was performed at the DNA sequencing Core Laboratory at the University of Florida (Gainesville, FL), using fluorescent dye termination sequencing chemistry on an Applied Biosystems 373A DNA sequencer. DNA sequences were analyzed using GeneWorks 2.2 (IntelliGenetics). The constructs were transferred into *Agrobacterium tumefaciens* EHA-105 by using a direct transformation protocol (An et al., 1988)

Plant Transformation

Arabidopsis ecotype Wassilewskija (Ws) plants transformed with 35S-anti-mu and 35S-chi-NT genes were obtained by using a vacuum infiltration transformation method (Bechtold et al., 1993; Bihn, 1997).

RNA Analysis of Wild-Type (Wt) and Transgenic Arabidopsis Plants

Total RNA was extracted using TRIzol® reagent (BRL) following manufacturer's instructions. Gel blot analyses were performed according to protocol by Amersham. The cDNA of GF14 mu was cloned into pCR®-Blunt Vector (Invitrogen). Gene- and strand-specific probe of GF14 mu was synthesized using MAXIscript™ kit (Ambion) according manufacturer's instructions. Hybridization was performed at 67 ^{0}C for 18 h in 0.5 M sodium phosphate, pH7.2, 1 mM EDTA, 7% SDS, and 1% BSA. The blots were washed for 1 h with four changes of 40 mM sodium phosphate, pH 7.2, 24 mM NaCl, 0.1% SDS, and 1mM EDTA at 69 ^{0}C, and exposed to a Phosphor Screen (Molecular Dynamics), and the images and quantitative analysis of the blots were processed using Image Quant program (Molecular Dynamics). Full-length GF14 chi probe was synthesized using random prime labeling kit from Boehringer. Gene-specific probe of GF14 chi was synthesized from the Sca I and EcoR I fragment of GF14 cDNA in a similar manner as that of full-length.

Results and Discussion

Expression Pattern of GF14 mu and chi in WT Arabidopsis (Ws)

To examine the expression pattern of the Arabidopsis 14-3-3 mu and chi isoforms, total RNA was extracted from different tissues of Arabidopsis plants (Ws ecotype). Northern blot analysis was subsequently performed. As shown in Figure 4-1, GF14 mu was predominantly expressed in leaves and stems and trace amount of message was

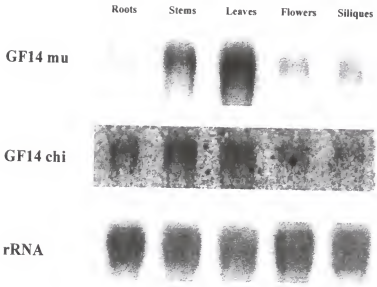


Figure 4-1. Expression pattern of GF14 mu and GF14 chi in Arabidopsis (Ws ecotype). Total RNA was extracted from roots, stems, leaves, flowers, and siliques of green-house grown Arabidopsis plants as described in Materials and Methods. The RNA blot was hybridized with the full-length cDNA of GF14 mu and GF14 chi gene-specific probe as described in Materials and Methods. 28S rRNA was used as the control to ensure equal loading of the lanes.

present in roots, flowers, and siliques. Similar results was obtained using a GF14 mu gene-specific probe. GF14 chi showed strong expression at stems, leaves, and roots and weak expression in flowers and siliques. Thus, GF14 mu and chi display tissue-preferential expression pattern, indicating that they may encode for distinct functions. The expression pattern of GF14 chi in Ws Arabidopsis differs from that in Columbia ecotype, which shows strong expression in roots and reproductive organs, while weak expression in the vegetative tissues (Daugherty et al., 1996). These results suggested that GF14 chi in Arabidopsis Ws ecotype may play a different role compared to that in Columbia ecotype.

In Arabidopsis, certain genes associated with pathogen attack resistance (Aufsatz and Grimm, 1994; Kunkel et al., 1993) also possess ecotype-specific expression pattern. And this expression pattern strongly argues against GF14 chi's functions in development or metabolism, but perhaps suggests GF14 chi's involvement in signal transduction response pathways.

Transgenic Arabidopsis Overexpressing Antisense GF14 mu

To investigate the functions of GF14 mu, Arabidopsis plants (Ws ecotype) were transformed with vector carrying the fusion of CaMV 35S promoter and GF14 mu in the antisense orientation (Figure 4-2). Thirty transgenic plants were generated as described in Materials and Methods. Transgenic lines of T_3 generation homozygous for the transgene were selected for further analysis. The presence of the transgene was indicated by the expression of neomycin phosphotransferase, which confers the kanamycin resistance. Wt

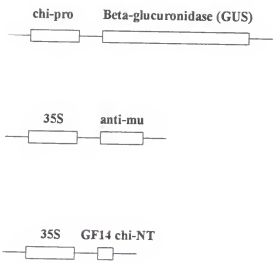


Figure 4-2. Schematic illustration of plasmid constructs used in the transgenic Arabidopsis. Constructs were made as described in Materials and Methods.

plants and transgenic plants carrying a GF14 chi promoter GUS fusion transgene (chi-pro-GUS) (Daugherty et al., 1996) were included as controls.

Arabidopsis plants containing antisense mu transgene were phenotypically indistinguishable from Wt and chi-pro-GUS transgenic control plants in the aerial parts. Plant height, flowering time, leaf number and leaf size of plants grown under the same greenhouse conditions were unaltered (Figure 4-3, only anti-mu-1 is shown).

To detect whether and to what extent the endogenous mRNA level was altered due to the expression of antisense mu transgene, RNA gel analysis was performed. The results are shown in Figure 4-4A (only anti-mu-1 is shown). In anti-mu-1, the GF14 mu message level was reduced to approximate 42% of that of Wt plants.

Transgenic Arabidopsis with Overexpression of GF14 chi Dimerization Domain

Results from previous studies indicated that the N-terminal half regions of 14-3-3 proteins function as the dimerization domains while the C-terminal regions are responsible for interacting with other proteins. And the 14-3-3 dimers are critical for at least some of their normal functions. Therefore, overexpression of the dimerization domain of one 14-3-3 isoform *in vivo* may generate substantial amount of "defective" heterodimers which may in turn induce significant disruption of endogenous 14-3-3 functions and/or localization.

To test this hypothesis, cDNA sequence encoding amino acid 1 through 139 of GF14 chi (chi-NT) was cloned in sense orientation under the control of 35S CaMV promoter (35S-chi-NT) (Figure 4-2). Subsequently, 35S-chi-NT was transformed into Arabidopsis plants (Ws ecotype) as described in Materials and Method. The transgenic lines of the T_2 generation (30 lines) were selected for further analysis. The presence of

Figure 4-3. Transgenic Arabidopsis plants with anti-mu, 35S-chi-NT are phenotypically indistinguishable from the Wt and chi-pro-GUS transgenic control plants. Arabidopsis plants were grown under constant light for 30 days.

WT

chi-pro-GUS

anti-mu-1

35S-chi-NT-24

35S-chi-NT-20

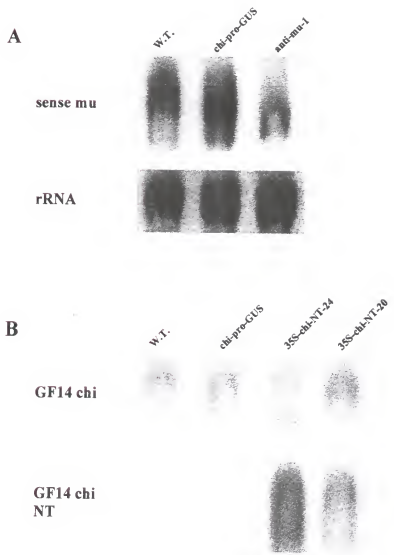


Fig4-4. Transgene Expression.
A. RNA gel blot analysis of the transgenic Arabidopsis with antisense GF14 mu. Total RNA was extracted from leaves of 3 week plants and and gene-and strand-specific probe were prepared as described in Materials and Methods. Wild type and transgenic plant with GF14 chi-pro-GUS were included as control.
B. RNA gel blot analysis of the transgenic Arabidopsis with overexpression of the dimerization domain (NT) of GF14 chi. RNA samples were prepared as described above. RNA blot was hybrized with full-length GF14 chi probe.

transgene was indicated by the expression of neomycin phosphotransferase and the presence of mRNA corresponding to the chi-NT (see below).

Similar to transgenic plants overexpressing antisense GF14 mu, the transgenic Arabidopsis plants expressing chi-NT displayed no detectable phenotypic changes compared to Wt or chi-pro-GUS transgenic controls (Figure 4-3). Hence, RNA was extracted from the leaves of the transgenic and Wt plants and northern blot was performed to detect the expression level of the 35S-chi-NT transgene. As shown in Figure 4-4B, the 35S-chi-NT transgene expression was not present in either Wt or transgenic chi-pro-GUS control plants, whereas it was present in the two 35S-chi-NT transgenic plants as expected. At present, I am unable to examine the protein expression level of chi-NT due to the lack of antibody against the N-terminal region of GF14 chi isoform.

The reason why transgenic plants with overexpression of antisense GF14 mu failed to induce macroscopic morphological changes in Arabidopsis may be several fold.

First, the reduction of GF14 mu mRNA may have minimal impact on its protein expression level. At present I cannot exclude this possibility because of the lack of mu-specific antibody and/or method of analyzing mu-specific activity. Second, if GF14 mu protein expression is reduced, it is possible that this reduction has little influence on the cellular functions of Arabidopsis, which may either be a result of its own functional insignificance or functional redundancy of other isoforms. Third, reduced expression of GF14 mu may induce metabolic changes at subcellular level which may have minimal impacts to macroscopic morphology, like that of antisense cytosolic fructose-1,6-bisphosphatase in transgenic potato plants (Zrenner et al., 1996).

Similarly, 35S-chi-NT transgenic plants may also induce changes at subcellular level However these disruptions seem not able to cause dramatic gross morphological alterations. Therefore, further examination of these transgenic plants, such as NR activity, fusicoccin perception and related activities, and G-box binding factor related activities, may still lend insight to the functional roles of Arabidopsis 14-3-3 proteins.

CHAPTER 5
SUMMARY

14-3-3 proteins have been indicated to be involved in diverse signal transduction pathways in many eukaryotic organisms. Although the exact functions of these highly homologous proteins remain unknown, a certain degree of consensus has been forming. First, 14-3-3 proteins from diverse origins appear to share similar, if not identical, fundamental property that is the ability to interact with different proteins with similar 14-3-3-binding motifs (chapter 1). Second, the interactions with 14-3-3s by different proteins leads to diverse results. Through binding to 14-3-3 proteins, some proteins, such as Raf-1, Bcr and A20 which are protein kinases and/or kinase substrates, may come to proximity where phosphorylation or other modifications may occur. Some proteins, like Raf-1 and nitrate reductase (NR), may stabilize their phosphorylated state upon interacting 14-3-3 proteins, which in turn results in either activation or inhibition of these proteins, respectively. Third, 14-3-3 proteins seem to exist as protein families in all organisms investigated to date and different isoforms appear to possess isoform-preferential, if not isoform-specific, expression patterns.

Arabidopsis 14-3-3 proteins were first identified by screening an expression library using antibodies raised against component(s) of G-box protein-binding complex. They were subsequently designated as GF14 (G-box Factor 14-3-3 homolog) However, later

experiments showed that GF14 proteins do not bind to G-box, indicating they may associate with G-box protein-binding complex through interacting with other proteins. In spinach, 14-3-3 homologs have been shown to be the inhibitor proteins of NR and Arabidopsis 14-3-3s can interchangeably carry out these binding and inhibitory activities. In oat and several other plant species, 14-3-3s were demonstrated to be part of phytotoxin fusicoccin receptor, linking 14-3-3 to receptor initiated signal transduction pathway in plants for the first time. Despite those discoveries, the exact functions of 14-3-3 proteins in Arabidopsis remain to be investigated.

Seven 14-3-3 isoforms have been identified in mammals. In Arabidopsis, sequences of six 14-3-3 cDNA, GF14 psi, chi, phi, omega, upsilon, and RCI 2 (**R**apid **C**old-**I**nducible cDNAs) and three genomic clones, GRF1-GF14 chi, GRF2-GF14 omega, GRF3-GF14 psi (for **G**eneral **R**egulatory **F**actor, with numbers designating the order in which the genomic clones were identified) have been identified. Low-stringency Southern blot analysis indicated that Arabidopsis contains more than six 14-3-3 homologs (Wu et al., 1997b).

The objective of this study is to identify all members of Arabidopsis 14-3-3 homologs in Arabidopsis and biochemically and functionally characterize these proteins.

I took two approaches to clone the new members of Arabidopsis 14-3-3 gene family. First, I screened an Arabidopsis yeast two-hybrid library using GF14 chi, one of the previously identified isoforms, as probe. Seven positive clones encoding proteins that interacted with GF14 chi were isolated. Sequencing of those clones revealed three unique 14-3-3 sequences. Two of the sequences represent novel 14-3-3 proteins and they were designated as GF14 epsilon and GF14 kappa (Wu et al., 1997a). To keep the consistency

of nomenclature in our study, one of the sequences that is identical to RCI2 was designated GF14 lambda. Then, using eight known cDNA sequences of Arabidopsis 14-3-3s, I BLAST-searched the dbEST at Genbank to screen for novel 14-3-3 sequences. 17 out of 103 EST entries with high homology to 14-3-3 sequences were not identical to any of the probes used. Sequence analysis revealed the presence of two new 14-3-3 sequences. They were designated as GF14 mu and GF14 nu (Wu et al., 1997b). Thus, I identified four new members of Arabidopsis 14-3-3 protein family. Together with six previously described Arabidopsis 14-3-3 homologs, they constitute a complete family of ten distinct 14-3-3 proteins of 248 to 268 amino acids. Sequence analysis revealed the presence of two ancient, diverse 14-3-3 gene classes, epsilon-like and nonepsilon isoforms.

The sequences of two new genomic clones of Arabidopsis 14-3-3 genes, GRF4-GF14 phi and GRF5-GF14 upsilon, were also identified (Wu et al., 1997b). They shared the same three conserved intron positions as those of previously identified genomic clones. The grouping of these 14-3-3 genes (with known genomic structure) based on similarity of intron positions conform with that based on protein sequence analysis (Wu et al., 1997b).

To biochemically characterize Arabidopsis 14-3-3 proteins, five 14-3-3 proteins were expressed in an *E. coli* protein expression system. Gel filtration analysis indicated that they exist as dimers. Purified *E. coli* expressed 14-3-3 proteins can also heterodimerize with each other in a native gel system. This ability of homo- and heterodimerization eight Arabidopsis 14-3-3 isoforms and one maize isoform was also confirmed in a yeast two-hybrid system. The domains sufficient for dimerization with

different isoforms were identified in GF14 chi isoform. In addition, native gel western analysis also indicated the presence of heterodimer *in vivo* (Wu et al., 1997a).

To functionally characterize 14-3-3 proteins, I established two transgenic plants in which antisense GF14 mu (anti-mu) and GF14 chi dimerization domain (chi-NT) were introduced, respectively (chapter 4). In one of anti-mu transgenic plants, the endogenous GF14 mu mRNA level was reduced to approximate 42% of that of wild-type. In chi-NT transgenic plants, the mRNA for chi dimerization domain was overexpressed. However, neither transgenic plants displayed detectable gross morphological changes (chapter 4), indicating further detailed analysis is required to elucidate the nature and extent of the potential changes caused by those transgenes.

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BIOGRAPHICAL SKETCH

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